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GENETICS OF IRRITABLE BOWEL SYNDROME AND ASSOCIATED GASTROINTESTINAL SYMPTOMS

Maria Henström

Karolinska Institutet

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Genetics of Irritable Bowel Syndrome and Associated Gastrointestinal Symptoms

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By

Maria Henström

Principal Supervisor:
Prof. Mauro D'Amato
Ikerbasque Research Professor
Biodonostia Health Research Institute
San Sebastián, Spain
and
Visiting Professor of Gastrointestinal Genetics
Karolinska Institutet, Sweden
Department of Medicine Solna
Unit of Clinical Epidemiology

Co-supervisors:
Prof. Joseph Rafter
Karolinska Institutet, Sweden
Department of Biosciences and Nutrition

Dr. Mira Wouters
University Hospital Leuven, Belgium
Department of Clinical and Experimental Medicine
Translational Research Center for Gastrointestinal Disorders (TARGID)

Opponent:
Assoc. Prof. Åsa Torinsson Naluai
Sahlgrenska Academy at the University of Gothenburg, Sweden
Institute of Biomedicine
Department of Microbiology & Immunology

Examination Board:
Prof. Robert Brummer
Örebro University, Sweden
Faculty of Medicine and Health
Department of Medical Sciences
Nutrition-Gut-Brain Interaction Research Centre

Assoc. Prof. Elísabet Einarsdóttir
Karolinska Institutet, Sweden
Department of Biosciences and Nutrition

Prof. Alicja Wolk
Karolinska Institutet, Sweden
Institute of Environmental Medicine
Unit of Nutritional Epidemiology

Stockholm 2018

To my boys

Gustav, Tor and Sixten

You are my life and my everything

Jag älskar er ♥

Sixten "Sigge" (June-17) and Tor (Jan-16)

“The more you know, the more you know you don’t know.”

Aristotle

ABSTRACT

Irritable bowel syndrome (IBS) affects more than one in ten individuals worldwide and is the most common cause of gut illness. It is classified according to the symptom-based Rome criteria as a functional gastrointestinal disorder (FGID) characterized by recurrent abdominal pain or discomfort accompanied with altered bowel habits (diarrhea, constipation or both). Although the research interest in IBS has grown considerably lately and several contributing factors are being recognized, the etiology of IBS is still far from understood. Hence, there is no effective cure and no established biomarkers, and current therapeutic options can only be directed towards symptom amelioration. IBS significantly reduces peoples' quality of life and working ability, and has important socioeconomic consequences, posing a considerable burden both on the affected individual and the society at large. Therefore, there is an urgent need for improved understanding of this common gastrointestinal disorder.

A heritable component in IBS has been demonstrated although inadequately studied; hence not much is known about the specific genetic architecture of IBS. Through focused genetic research, we strive to gain novel insight into the pathophysiology of IBS. Eventually, these studies will contribute to shifting the paradigm from symptom-based definitions to a molecular re-classification of patients, for clinical translation and post-genomic approaches to stratified medicine in IBS.

The overall aim of this thesis was therefore to identify, validate and functionally characterize genetic factors predisposing to IBS and associated gastrointestinal symptoms. The first three papers included in this thesis are hypothesis- or pathway-driven candidate-gene studies investigating the role of specific genes in IBS predisposition, while the fourth paper is an important step in our broader approach to using large general population-based studies for IBS gene-hunting efforts.

In **paper I**, we showed that genetic variation in the *NPSR1* gene influences children's predisposition to recurrent abdominal pain (RAP), the cardinal symptom of IBS and related FGIDs. The *NPSR1* gene encodes the neuropeptide S (NPS) receptor, and previous evidence suggests its signaling to influence functions along the brain-gut axis, including mucosal immune activity, secretion of other neuropeptides and gut hormones, pain perception, and gut motor and sensory functions.

In **paper II** we aimed to investigate the role of ion channel genes in IBS risk. By genotyping a Swedish case-control cohort for four genes that showed nominal significance in our previously published pilot genome-wide association study (GWAS) of IBS, we could provide evidence of association for the transient receptor potential cation channel gene *TRPM8* (the 'cold and menthol receptor'). *TRPM8* polymorphisms showed significant association with constipation-predominant IBS subtypes (IBS-C/M), and risk alleles further correlated with harder stool consistency in an independent population-based Swedish dataset.

In **paper III**, through a series of experiments and association analyses, we could demonstrate a potential mechanism underlying the often perceived link between carbohydrate consumption and IBS symptoms. Rare sucrase-isomaltase *(SI)* mutations were more common in IBS patients compared to controls or the general population, and we also provide evidence of a functionally relevant coding SNP that significantly increases the risk of IBS, especially diarrhea-predominant IBS. This study suggests that milder forms of genetically derived SI deficiency may be present in subgroups of patients currently classified as IBS.

Finally, **paper IV** represents an important step in an alternative strategy for the identification of IBS risk genes and variants; a general population-based approach utilizing existing data in large epidemiological cohorts and biobanks. Here we conducted a meta-analysis of a total of 1,335 IBS cases and 9,768 asymptomatic controls from five independent European GWA studies, and although no genome-wide significant association was detected, the results from this study identify seven suggestive IBS risk loci for further investigation and highlight ion channel activity as potentially implicated in IBS pathophysiology.

In conclusion, elucidating the genetic architecture of IBS is a truly challenging task, but as we are making progress, the studies in this thesis represent a significant step forward. We provide evidence for the importance of specific genes (*NPSR1*, *TRPM8* and *SI*) in the development of IBS and associated phenotypes in subsets of patients, contribute to the emerging evidence suggesting a role of ion channels in IBS pathophysiology, and confirm the applicability of using large general-population based cohorts for the discovery of IBS risk genes and variants.

LIST OF SCIENTIFIC PAPERS

I. **Henström M**, Zucchelli M, Söderhäll C, Bergström A, Kere J, Melén E, Olén O, D'Amato M.
NPSR1 polymorphisms influence recurrent abdominal pain in children: a population-based study.
Neurogastroenterol Motil 2014;26(10):1417-25.

II. **Henström M**, Hadizadeh F, Beyder A, Bonfiglio F, Zheng T, Assadi G, Rafter J, Bujanda L, Agréus L, Andreasson A, Dlugosz A, Lindberg G, Schmidt PT, Karling P, Ohlsson B, Talley NJ, Simrén M, Walter S, Wouters M, Farrugia G, D'Amato M.
TRPM8 polymorphisms associated with increased risk of IBS-C and IBS-M.
Gut 2017;66(9):1725-7.

III. **Henström M**, Diekmann L, Bonfiglio F, Hadizadeh F, Kuech EM, von Köckritz-Blickwede M, Thingholm LB, Zheng T, Assadi G, Dierks C, Heine M, Philipp U, Distl O, Money ME, Belheouane M, Heinsen FA, Rafter J, Nardone G, Cuomo R, Usai-Satta P, Galeazzi F, Neri M, Walter S, Simrén M, Karling P, Ohlsson B, Schmidt PT, Lindberg G, Dlugosz A, Agréus L, Andreasson A, Mayer E, Baines JF, Engstrand L, Portincasa P, Bellini M, Stanghellini V, Barbara G, Chang L, Camilleri M, Franke A, Naim HY, D'Amato M.
Functional variants in the sucrase-isomaltase gene associate with increased risk of irritable bowel syndrome.
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IV. Bonfiglio F, **Henström M**, Nag A, Hadizadeh F, Zheng T, Cenit MC, Tigchelaar E, Williams F, Reznichenko A, Ek WE, Rivera NV, Homuth G, Aghdassi AA, Kacprowski T, Männikkö M, Karhunen V, Bujanda L, Rafter J, Wijmenga C, Ronkainen J, Hysi P, Zhernakova A, D'Amato M.
A GWAS meta-analysis from five population-based cohorts implicates ion channel genes in the pathogenesis of irritable bowel syndrome.
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PUBLICATIONS NOT INCLUDED IN THE THESIS

I. **Henström M**, D'Amato M.
Genetics of irritable bowel syndrome.
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LIST OF ABBREVIATIONS

ANS	Autonomic nervous system
BA	Bile acid
BAM	Bile acid malabsorption
BAMSE	Children Allergy Milieu Stockholm and Epidemiological Study
BM	Bowel movement
BSFS	Bristol stool form scale
CI	Confidence interval
CNS	Central nervous system
CSID	Congenital sucrase-isomaltase deficiency
EMR	Electronic medical records
ENS	Enteric nervous system
eQTL	Expression quantitative trait loci
EUR	European population
ExAC	Exome Aggregation Consortium
FBD	Functional bowel disorder
FC	Functional constipation
FDr	Functional diarrhea
FDR	False discovery rate
FGF19	Fibroblast growth factor 19
FGID	Functional gastrointestinal disorder
FODMAPs	Fermentable Oligo-, Di- and Monosaccharides And Polyols
GI	Gastrointestinal
GO	Gene Ontology
GSEA	Gene-set enrichment analysis
GTEx	Genotype-Tissue Expression project
GWAS	Genome-wide association study
HPA	Hypothalamic-pituitary-adrenal
HWE	Hardy–Weinberg equilibrium
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome

IBS-C	Constipation-predominant IBS
IBS-D	Diarrhea-predominant IBS
IBS-M	Mixed IBS
IBS-U	Unclassified IBS
ICC	Interstitial cells of Cajal
ICD	International Classification of Diseases
LD	Linkage disequilibrium
MAF	Minor allele frequency
MGAM	Maltase-glucoamylase
NCGS	Non-coeliac gluten sensitivity
NFBC1966	Northern Finland Birth Cohort 1966
NPS	Neuropeptide S
NPSR1	Neuropeptide S receptor 1
OR	Odds ratio
PI-IBS	Post-infectious IBS
PopCol	Population-based Colonoscopy study
QC	Quality control
RAP	Recurrent abdominal pain
RCT	Randomized controlled trial
SALT	Screening Across the Lifespan Twin survey
SCFA	Short-chain fatty acid
SHIP	Study of Health in Pomerania
SI	Sucrase-isomaltase
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variations
SSRI	Selective serotonin re-uptake inhibitor
TNF	Tumor necrosis factor
TRP	Transient receptor potential
TRPM8	Transient receptor potential cation channel melastatin 8
TSEA	Tissue-specific enrichment analysis

1 BACKGROUND

1.1 IRRITABLE BOWEL SYNDROME: THE CLINICAL ENTITY

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder and the most common cause of gut illness. IBS is not a dangerous condition per se, that is to say, it is not a life-threatening disease. Nevertheless, it is definitively a serious matter. It is extremely common, has a high economic burden, and reduces peoples' quality of life and working ability. At the same time, the etiology is poorly understood and established diagnostic biomarkers are absent; hence there is no specific cure. Therefore, there is an urgent need for improved understanding of this gastrointestinal disorder, and while the interest in IBS research has grown considerably lately, increasing hope has been put in genetic studies.[1-6] By elucidating the genetic background of IBS we will gain important knowledge of its etiology, contribute to re-classification of this condition into subgroups based on underlying pathophysiology rather than symptoms, and identify pathways and biological targets that can be exploited for therapeutic purposes.

1.1.1 Clinical appearance

While the term *organic disease* describes a condition explained by structural or biochemical abnormalities of tissues, organs or systems of the body, instead, a *functional* condition relates to the presentation of symptoms without detectable organic explanations. In gastroenterology, a large portion of diagnoses is in fact functional. The first three leading symptoms prompting a visit to outpatient clinics in the U.S. in 2009 were abdominal pain (15.9 million visits), diarrhea (4.2 million) or constipation (3.2 million),[7] and between 35% and 45% of patients seen in gastroenterology clinics will ultimately be diagnosed with *functional gastrointestinal disorders* (FGIDs).[8, 9] With symptoms arising from different parts of the gastrointestinal (GI) tract, FGIDs constitute a group of GI disorders classified based on patients' symptomatology according to diagnostic criteria developed by the Rome Foundation,[10] and in "the absence of obvious anatomic or physiologic abnormalities identified by routine diagnostic examinations, as deemed clinically appropriate".[11] Based on this classification system, FGIDs in adults are divided into six subgroups depending on the location of the main symptoms. Irritable bowel syndrome (IBS) (**Figure 1**) belongs to the functional bowel disorders (FBD) —one of these six FGID groups. IBS is by far the most common reason for seeing a gastroenterologist, and also constitutes 12% of patients seeking medical attention in primary care practices.[12]

The clinical presentation of IBS is characterized by recurrent abdominal pain or discomfort ("an uncomfortable sensation not described as pain")[13] in combination with alterations in bowel habits. Based on patients' predominant stool pattern, IBS can be subtyped as diarrhea-predominant (IBS-D), constipation-predominant (IBS-C) or mixed/alternating IBS (IBS-M).[11, 13] Patients who cannot be accurately categorized in one of these groups are assigned IBS-U (unclassified). The stool pattern is assessed using Bristol Stool Form Scale (BSFS; **Figure 1C**), which is a scale from 1 to 7 describing the stool consistency. The BSFS is a reliable surrogate marker for intestinal transit time (see further info in section **1.3**); hence,

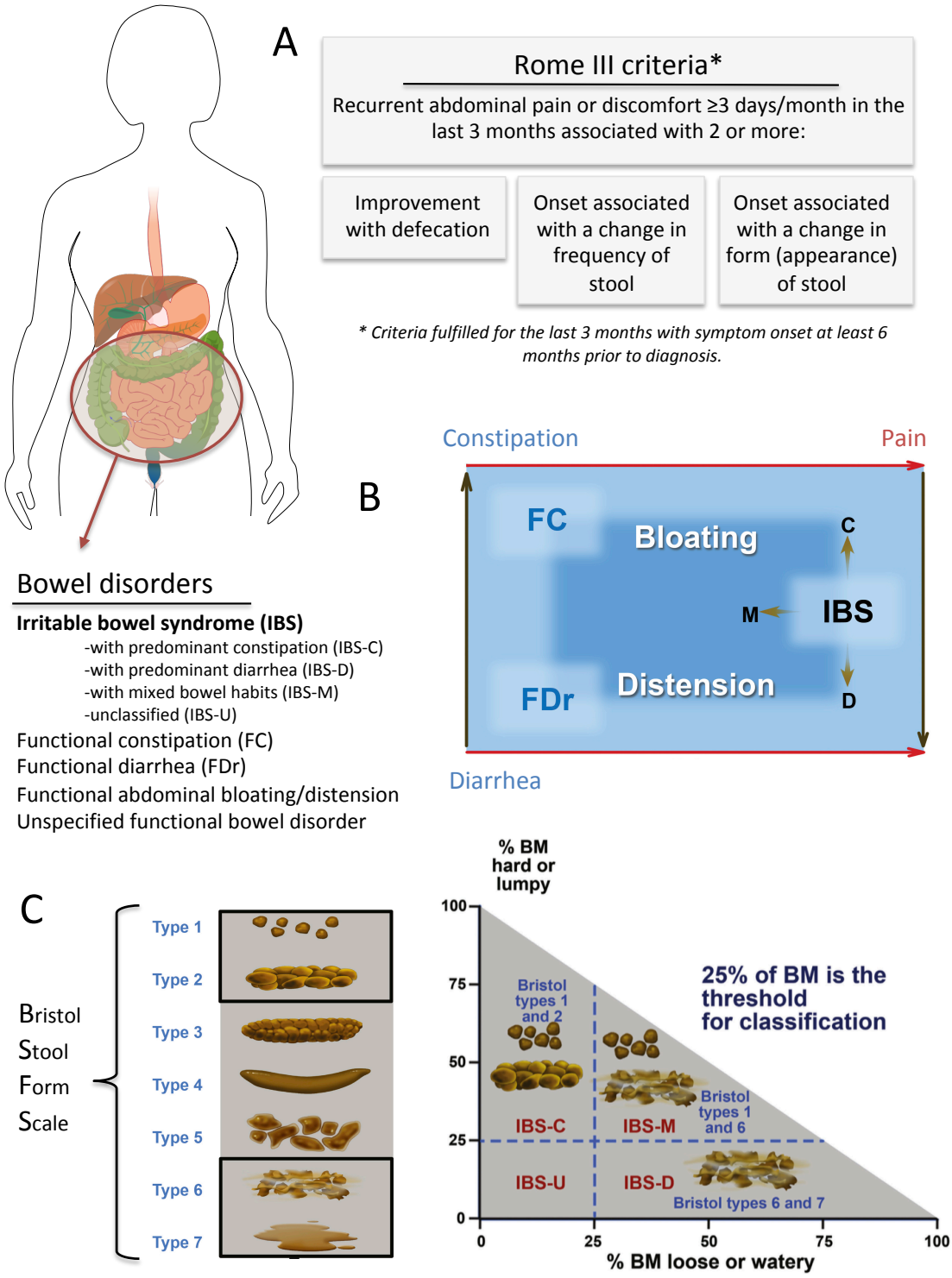


FIGURE 1. Rome III criteria classification of IBS and its subtypes. Functional gastrointestinal disorders (FGIDs) are classified into six groups based on predominant symptom location. One of these is the functional bowel disorders (FBDs), which includes IBS. *(A)* Rome III diagnostic criteria for IBS. *(B)* Conceptual framework to illustrate the significant overlap that exists between the FBD conditions, which should be considered to be part of a spectrum rather than separate, isolated entities. Pain, diarrhea and constipation distinguish IBS, FC and FDr from each other, while bloating and distension are symptoms frequently reported by patients with any of the FBDs. *(C)* Subclassification of IBS using the Bristol stool form scale (BSFS), a reliable surrogate marker for intestinal transit time, to evaluate the consistency of patients' bowel movements (BMs). *(B/C)* Lacy *et al.*,[11] © see page 71.

this is a useful tool to evaluate abnormal bowel habits. Scores 1 and 2 indicate constipation pattern, 6 and 7 diarrhea, and 3-5 are considered normal types of stool. With ≥25% hard or lumpy stools (i.e., 1 or 2) and <25% loose or watery stools (6 or 7), patients are classified as IBS-C. Conversely, having ≥25% loose or watery stools and <25% hard or lumpy stools classifies as IBS-D. Patients with both ≥25% hard or lumpy stools and ≥25% loose or watery stools are placed in the IBS-M group. By capturing type, frequency and duration of symptoms, the Rome criteria were introduced in 1990 with the aim to separate healthy everyday GI symptoms from gut dysfunction,[14] and have been updated three times since; 1999-2000,[15] 2006,[13] and the latest Rome IV as recently as 2016.[11] Obviously, since Rome IV is very new, studies published to date, including those in this thesis, have used previous versions for phenotype definition (often III). Compared to Rome III (**Figure 1A**), the IV update contains a few revisions for IBS including the frequency of symptoms (on average ≥1 day/week) and rephrasing of the criteria (the terms *'discomfort'* and *'onset'* removed, and *'Improvement with'* changed to *'Related to'*), overall making it a bit more restrictive. In addition, while subtyping according to Rome III is based on all bowel movements (including normal ones),[13] the Rome IV criteria assess only days that contain symptomatic stools (i.e., loose/watery or hard/lumpy), attempting to reduce the unclassified subgroup.[10]

Although not actually part of the Rome criteria for IBS, bloating is also commonly experienced and reported by more than 80% of patients,[16] and is thus considered a supportive symptom that increases the confidence in the diagnosing of IBS.[13] Overall, there is a significant overlap between IBS and other FBDs,[17] and over time people tend to migrate between these, as well as between different IBS subtypes.[18-21] Hence, while it may not always be possible to confidently separate these conditions as discrete entities, they should also be considered part of a spectrum (**Figure 1B**).[11] In addition, other functional gastrointestinal disorders, such as reflux and dyspepsia, associate with IBS,[17, 22] as well as several non-GI comorbidities, including pain syndromes (fibromyalgia, chronic pelvic pain, chronic fatigue syndrome, and migraine) and psychiatric conditions (depression, anxiety and somatization).[23-26] Moreover, a number of gastrointestinal diseases or conditions, e.g., celiac disease, inflammatory bowel disease (IBD), microscopic colitis or carbohydrate intolerance, can present with similar symptoms as in IBS and might therefore be misclassified as IBS if not detected in clinical examinations, or potentially delayed in their diagnosis.[27, 28] The Rome Foundation encourages positive diagnosing of IBS, which means applying symptom-based criteria and performing limited diagnostic testing (such as colonoscopy, fecal calprotectin and blood tests) only in case of alarm signs (e.g., blood in the stool, unintentional weight loss, or family history of colorectal cancer or IBD).[29, 30] However, in practice, IBS is more often considered a 'diagnosis of exclusion'.[31]

The most well documented risk factor for IBS is gender, as it manifests predominantly in women.[32, 33] A comprehensive meta-analysis including 56 studies from around the world showed a significantly higher pooled prevalence of IBS in women (14.0%) compared to men (8.9%) with an international odds ratio of 1.67 (95% CI: 1.53-1.82),[34] although significant heterogeneity between studies was observed. The incidence of IBS is not frequently reported,

but a U.K. study implemented in 123 general practices, utilizing medical registries, reported higher incidence in women (5.8 per 1,000) than in men (1.9 per 1,000).[26] Stratifying by age, most patients were diagnosed in their early or middle life, with the highest incidence rate in the age span 25-44 years. Other studies suggest IBS risk to decrease after the age of 50, but due to significant heterogeneity between studies, the effect of age is unclear.[35]

Irritable bowel syndrome has a significant and serious impact on sufferers' well-being and quality of life.[36] Only about one in four individuals meeting IBS criteria seek medical attention,[37, 38] but those who do, utilize health care extensively.[39, 40] Compared to undiagnosed individuals, those who consult a physician tend to report more severe symptoms, higher levels of anxiety and poorer quality of life.[37, 41] In an international survey from 2009, including 1,966 IBS patients diagnosed by a physician, respondents reported their IBS to cause restriction of usual activities on average 73 days (20%) of the year, and almost 13% of patients were not working because of their health.[40] In the same survey, patients would be willing to give up 25% of their remaining life (on average 15 years) and 14% would risk a 1 in 1,000 chance of death in order to receive a treatment that would make them symptom free. This illustrates the real severity of IBS, and consequently, it has remarkably significant repercussions on health and socioeconomic systems.[42, 43] The economic burden constitutes both direct (healthcare) and indirect costs. The latter involves societal costs related to education, social services and industry/work, including absenteeism (time off work) and presenteeism (reduced productivity whilst at work). However, estimating these is a truly challenging task, especially indirect costs, and together with the fact that a large number of undiagnosed IBS sufferers exist in the general population, and the unclear overall IBS prevalence, the total 'price tag' for IBS is not known. Reviewing previous attempts, variations in cost estimates are considerable. For example, direct cost (per IBS patient and year) may be between $742-$7,547 in the U.S. and £90-£316 in the U.K..[43] Indirect costs attributed to IBS are even harder to calculate but constitutes approximately two-thirds of the total, and may correspond to about 0.5% of annual national healthcare budgets.[42]

1.1.2 Worldwide prevalence

The prevalence of IBS around the world is considerably large, usually reported on average between 10-20%.[1, 38] In a meta-analysis from 2012 on compiled data from 260,960 individuals and 80 separate study populations around the world, Lovell and Ford[35] estimated a pooled prevalence of 11.2% (95% CI: 9.8-12.8%), see **figure 2.**[1] However, the prevalence reported by individual studies varies substantially, ranging from 1.1% (U.S. and Iran) to 45.0% (Pakistan) in this particular meta-analysis. Also in a more recent literature review published in 2017, IBS prevalence estimates span from 1.1% (studies from Iran and France) to 35.5% (Mexico),[33] and due to large heterogeneity between studies, also within countries, the authors concluded a pooled estimate would not be appropriate nor meaningful. This large variation in reported estimates could partly be explained by non-consistent criteria for IBS definition and methodological variance. For example, in addition to using different classification criteria (i.e., Manning[44] or Rome I/II/III[13-15]), the way of collecting data

(posted/electronic questionnaires, telephone interview or personal interviews) may also influence the response rates and results.[33] The studies used in these literature reviews all used questionnaires or interviews and symptom-based criteria to capture IBS prevalence in the general population. However, if one would instead count the diagnoses present in the clinics (utilizing for example electronic medical registries, or simply asking patients if they have been medically diagnosed by their clinician) the numbers would look different, as you would only capture those who seek medical attention for their illness.[37] Moreover, cross-cultural differences such as illness interpretation and reporting, attitude towards health care, dietary habits, environmental hygiene, microbiota composition variation, and genetic differences, are important aspects to consider when evaluating and comparing IBS between countries and cultures.[45]

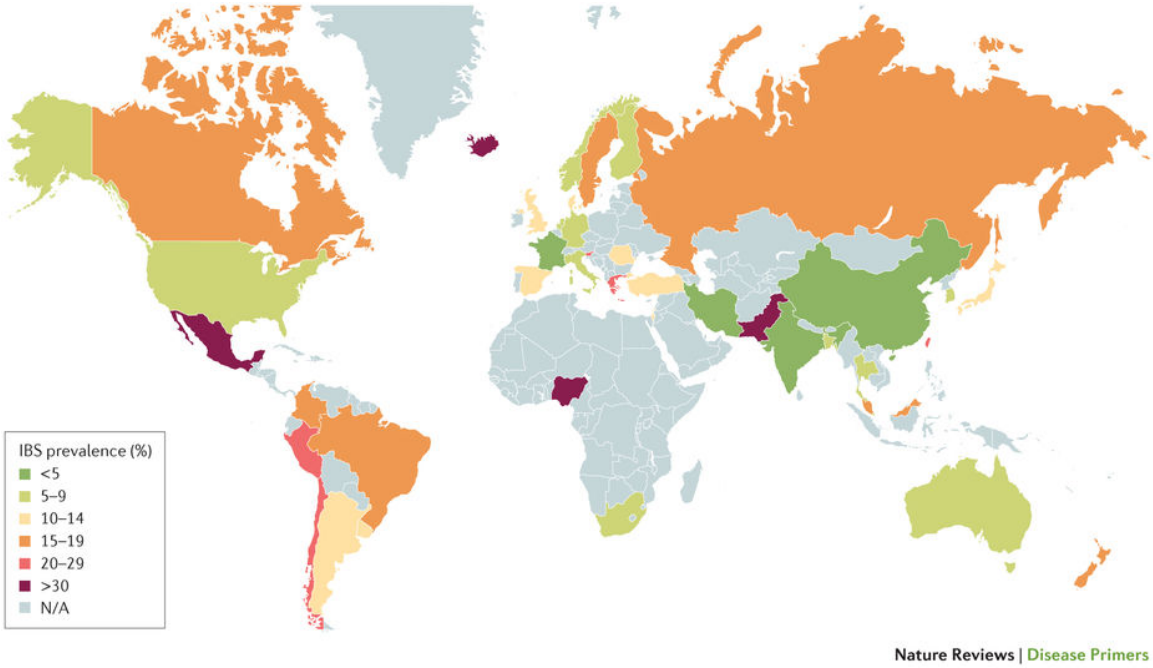


FIGURE 2. Worldwide prevalence of IBS. Data from population studies with results pooled by country and color-coded. Data from Lovell *et al.,*[35] supplemented by additional studies from another nine countries. Figure by Enck *et al.*,[1] © see page 71.

1.2 IBS PATHOPHYSIOLOGY

1.2.1 A disorder of gut-brain interaction

"Trust your gut feelings." "I've got butterflies in my belly."

We are all aware of the importance of our gut and its functions being closely linked to our mind and emotions, to the extent that it is even rooted in our everyday language. The relationship between our body and mind has been discussed for thousands of years and has had profound impact on the way science and medicine have developed.[10]

In the past, since IBS is difficult to explain in the absence of structural (organic) findings, and at the same time shows a strong psychological component, a common view has been that the

term *functional* may indicate an idiopathic or cryptogenic condition, with patients often labeled as neurotic and to suffer from a perceived illness although being otherwise healthy.[46] However, the concept of IBS being functional is about to change as we are learning more and more about the underlying mechanisms.[47] The development of more sophisticated methods now allows us to measure and detect 'real' abnormalities in IBS such as visceral hypersensitivity, inflammatory markers, gut microbiota composition, motility abnormalities and more, so it seems we are moving away from a purely symptom-based classification.[48] Although studies on IBS pathophysiology have been hampered by the lack of applicable biomarkers and the challenge in properly defining the IBS phenotype, the research interest in this area has remarkably expanded and there are now a number of factors and mechanisms proposed. We know today that psychological factors (e.g., stress, anxiety or depression) are indeed important in IBS,[23] but rather than being causative they may be considered risk factors (together with social conditions and early life events) that can exacerbate gastrointestinal symptoms and determine the severity of illness and the clinical outcome.[1, 10, 49] These psychosocial factors are part of a complex IBS pathophysiology model involving also environmental, genetic and physiological factors, such as mucosal immune activation, gut permeability, altered gut motility, visceral hypersensitivity, enteroendocrine and neuroimmune signaling, gut microbiota composition and activity, as well as food intolerance or carbohydrate malabsorption. This pathophysiology model can be described in different ways (as illustrated in several excellent reviews[1, 29, 47, 50-52]), and although the causality between involved factors is unclear, the overview picture tells us that IBS appears to be a condition with a brain-gut or gut-brain communication being somehow 'out of tune' (**figure 3**, page 10). Hence, —and to also move away from the concept of them being functional—, a re-definition of FGIDs has recently been proposed by the Rome Foundation; *Disorders of Gut-Brain Interaction.*[53]

Of note, IBS is not only a complex (multifactorial) disorder, but evidence also speaks for a highly heterogeneous phenotype in the sense that IBS should not be considered a single entity, but rather a mixture of various conditions all gathered under the same umbrella of similar symptoms. Furthermore, while the majority of IBS cases most likely have a multifactorial background with several contributing factors together causing disease onset, evidence also speaks for subsets of patients having specific underlying abnormalities, which on its own can induce IBS symptoms, such as in the case of bile acid malabsorption,[54] disaccharidase deficiencies,[55] or specific ion channelopathies.[56] Notably, this will also be reflected in their genetic predisposing background (see section **1.5** below).

1.2.2 Risk factors in IBS

As mentioned above, being female is the most obvious risk factor in IBS, as the condition is more prevalent in women than men in most countries worldwide.[33, 34] Other FGIDs also manifest predominantly in women, although the reason for this is not clear.[32] A role of sex hormones has been proposed, as they can modulate various processes that are related to IBS and the brain-gut axis, including pain processing, stress response, gut sensitivity and motility,

intestinal immune activation and barrier function. However, studies investigating the role of sex hormones in IBS are limited and difficult to conduct; hence, underlying mechanisms remain unknown.[32, 57] Age (>50 years of age) may be associated with a lower risk of IBS, but the relation between IBS and age is unclear.[35] Psychological factors and social environment (together referred to as psychosocial factors) such as anxiety, acute or chronic stress, illness behavior, coping, low quality of life, family history of substance abuse or mental illness, parents beliefs/behaviors, culture, socioeconomic status, social learning, social support, and early stressful life events, are all considered to influence the risk of IBS onset or severity.[1, 49] Moreover, presenting with other types of FGIDs, somatic pain syndromes (such as fibromyalgia, migraine or other), or psychiatric conditions, —which are all well-known comorbidities—, may also indicate higher risk of IBS, although it is unclear why these conditions co-exist.[1]

Infectious gastroenteritis (of bacterial, viral or other origin) is a risk factor for IBS, in particular IBS-D and IBS-M.[1] A systematic review and meta-analysis of eight studies showed a sevenfold increase in the odds of developing IBS after an infectious gastroenteritis (pooled OR 7.3 (95% CI, 4.7–11.1),[58] and a more recent systematic review including 45 studies (total n=21,421), found that at 12 months after an infectious gastroenteritis episode, 10.1% had developed IBS (relative risk 4.2; 95% CI, 3.1–5.7) and at more than 12 months the prevalence of IBS had increased to 14.5%.[59] Women are more likely than men to develop IBS after gastroenteritis, and other factors that seem to increase the risk include severity of the infection, psychological distress, depression, smoking and usage of antibiotics.[59, 60] Other prospective studies have estimated that between 3–36% of GI infections lead to IBS,[60] and a retrospective study reported that between 6–17% of IBS cases believe their IBS began with an acute gastroenteritis,[61] and these patients are referred to as post-infectious IBS (PI-IBS).

Finally, an underlying genetic predisposition exists in IBS, which can explain why some individuals are more susceptible to develop symptoms than others, and proposed risk genes play a role in several of the pathophysiological mechanisms presented below. However, the genetic background of IBS is the main focus of this thesis; hence it will be further discussed in detail in section **1.5**.

1.2.3 Pathophysiological factors

Abnormal GI motility and ***visceral hypersensitivity*** (as defined by an increased sensation of stimuli from the gut) are important features of IBS, and early research on IBS pathophysiology in the mid–20th century was centered on these two specific entities.[10] However, assuming that these gut motor and sensory abnormalities arise due to specific underlying etiological factors, these features can also be seen as intermediate phenotypes (and potential endophenotypes) in between the IBS manifestation and its underlying pathophysiological mechanisms (see **figure 3**, and **1.3** below). The brain is responsible for receiving and processing incoming sensory information from the periphery, and has an important role in the modulation of pain response by either amplifying or suppressing the signal. This endogenous pain modulation includes noradrenergic, serotonergic, opioidergic,

dopaminergic and cannabinoid pathways, which facilitate or inhibit neuronal activity in the spinal dorsal horn.[62] Pain perception is also highly influenced by mood (e.g., stress, depression or pleasure) and cognitive factors (e.g., expectations, memory and coping), and in addition, nociceptors (pain sensing nerve fibers) can be sensitized peripherally by for example injury or inflammation.[62] There is evidence now demonstrating impaired central pain processing in IBS, and even existence of structural differences in the central nervous system (CNS) in some patients.[52, 62, 63] However, through the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis the brain can also have effects on the gut,[64] and proposed ***'brain-to-gut'*** pathways in IBS suggest anxiety and prolonged stress (together with environmental, psychosocial and genetic factors) to directly or indirectly influence gut motility and secretion, barrier function, microbiota composition as well as mucosal immune response, which together lead to altered gut function and hypersensitivity.[1, 29, 50, 52] However, this brain-gut communication is very much bidirectional,[52] and evidence from epidemiological studies suggests that in about half of IBS sufferers, GI symptoms arise first and psychiatric/mood comorbidities develop later, indicating ***'gut-to-brain'*** pathways.[29, 47] Overall, the causality of events leading to hypersensitivity and other features of IBS is not clear, but several contributing factors have been proposed (further described below), involving both 'top-down' and 'bottom-up' mechanisms (**figure 3**).[1, 29, 47, 50, 52]

The intestinal barrier is the epithelial lining of the gut, which constitutes an enormous surface with direct contact with the luminal environment, and plays a crucial role in the host-microbe talk as a link between gut microbiota and the mucosal immune cells and enteric nervous system.[65, 66] This barrier is recognized as being of importance in several diseases, including IBD and celiac disease, but also IBS, and increased permeability (or 'leaky gut') has been described especially in IBS-D and PI-IBS.[65-68] Evidence suggests that environmental factors such as GI infection, medications, food components and the gut microbiota, all can cause increased intestinal permeability. At the same time, a permeable gut may allow more passage of bacterial or dietary antigens from the lumen across the mucosa, which is likely to facilitate a mucosal immune response with increased infiltration of inflammatory cells and mediators. This may in turn have effects on the enteric nervous system and neuromuscular function (causing dysmotility and diarrhea) and the upstream signaling to CNS (affecting pain perception and hypersensitivity, psychological well-being and behavior).[29, 50, 65, 66, 68]

Observations that support the role of ***immune activation*** in IBS include increased risk of IBS after gastroenteritis,[59] and the observation of IBS-like symptoms in IBD patients in remission.[69] Several studies have indicated increased numbers and activity of immune cells infiltrated in the mucosa of the GI tract of IBS patients, especially mast cells,[70] —a key component of the innate immune system. Mast cells in the GI mucosa lie close to sensory nerves, and signal to these using pro-inflammatory mediators (histamine, serotonin, proteases and others), causing peristalsis and pain responses, and possibly also sensitization.[70, 71] At the same time, efferent neurons can also activate mast cells through the release of neuropeptides and other transmitters. This is one example of ***neuroimmune signaling***; a complex bidirectional interaction between enteric neurons, terminals from extrinsic (ANS) nerves,

immune cells of the gut, and their mediators, which may contribute to increased permeability, changes in gut motility, and increased visceral pain perception in a subset of IBS patients.[1, 70] Interestingly, supernatants from colonic mucosal biopsies of IBS patients, but not from healthy controls, can activate enteric sensory neurons,[72] and this effect is stronger when supernatants from hypersensitive, rather than from normosensitive, patients are used.[73]

Neurotransmitters and neuropeptides are important in the brain-gut axis and have been implicated in IBS and associated GI symptoms and functions, with much attention being paid to ***serotonin*** (5-hydroxytryptamine, 5-HT).[1, 47, 51, 74] This monoamine is an important neurotransmitter involved in various processes including the regulation of mood, behavior and well-being, but also has significant effects on gut motility, secretion and visceral sensation, with more than 90% of serotonin being produced in the enterochromaffin cells in the GI tract.[75] Genetic studies, the effects from drugs targeting serotonin pathways on IBS symptoms and intestinal transit time, and observations of abnormal levels and impairment of serotonin metabolism and/or reuptake in IBS-D and IBS-C, all support the hypothesis of a dysregulated serotonin signaling in IBS.[74-76] Moreover, previous work from our group has provided evidence of the involvement of another neurotransmitter, neuropeptide S, and its receptor in gut motor and sensory functions[77] (further described in **1.5.5** below), which led us to perform **paper I** included in this thesis.

Ion channels are transmembrane proteins found in all types of cells where they coordinate the passage of ions in response to different stimuli. They often have cell-specific functions and in the GI tract ion channels play important roles in gut functions such as GI motility, secretion and visceral sensation.[78, 79] Channelopathies, i.e., dysfunctions (typically genetically derived) in conducting ions, are well established phenomena contributing to disease in the fields of neurology and cardiology, but are now also being increasingly recognized as important in the pathophysiology of FGIDs, in particular IBS.[74, 80] For instance, about 2% of IBS patients, mainly IBS-C, harbor loss-of-function mutations in a voltage-gated sodium channel (encoded by the *SCN5A* gene) important for smooth muscle cell contractility and the slow wave function initiated by interstitial cells of Cajal (ICC) in the gut.[56] There is also a growing body of evidence supporting the role of transient receptor potential (TRP) channels in IBS, especially in visceral pain perception.[81, 82] The TRPV1 channel, a thermo TRP, has been particularly recognized and is believed to contribute to hypersensitivity and pain in IBS, possibly through sensitization of sensory nerves by mast cell mediators.[70, 83] In **paper II** in this thesis, we also provide evidence of the potential involvement of another TRP channel (*TRPM8*) in IBS. Furthermore, recent work from our group in collaboration with A Zhernakova (University of Groningen) suggests ion channel activity to be implicated in gut function, based on the results from a first GWAS of stool frequency.[84] Of note, many of the currently available pharmacological treatments for IBS, e.g., prosecretory agents such as Lubiprostone and Linaclotide (see **1.4** below), exert their action through activation or inhibition of ion channels, and with the increasing body of evidence on the importance of ion channels in IBS pathophysiology, these represent reachable and promising targets for IBS therapy.[78, 80]

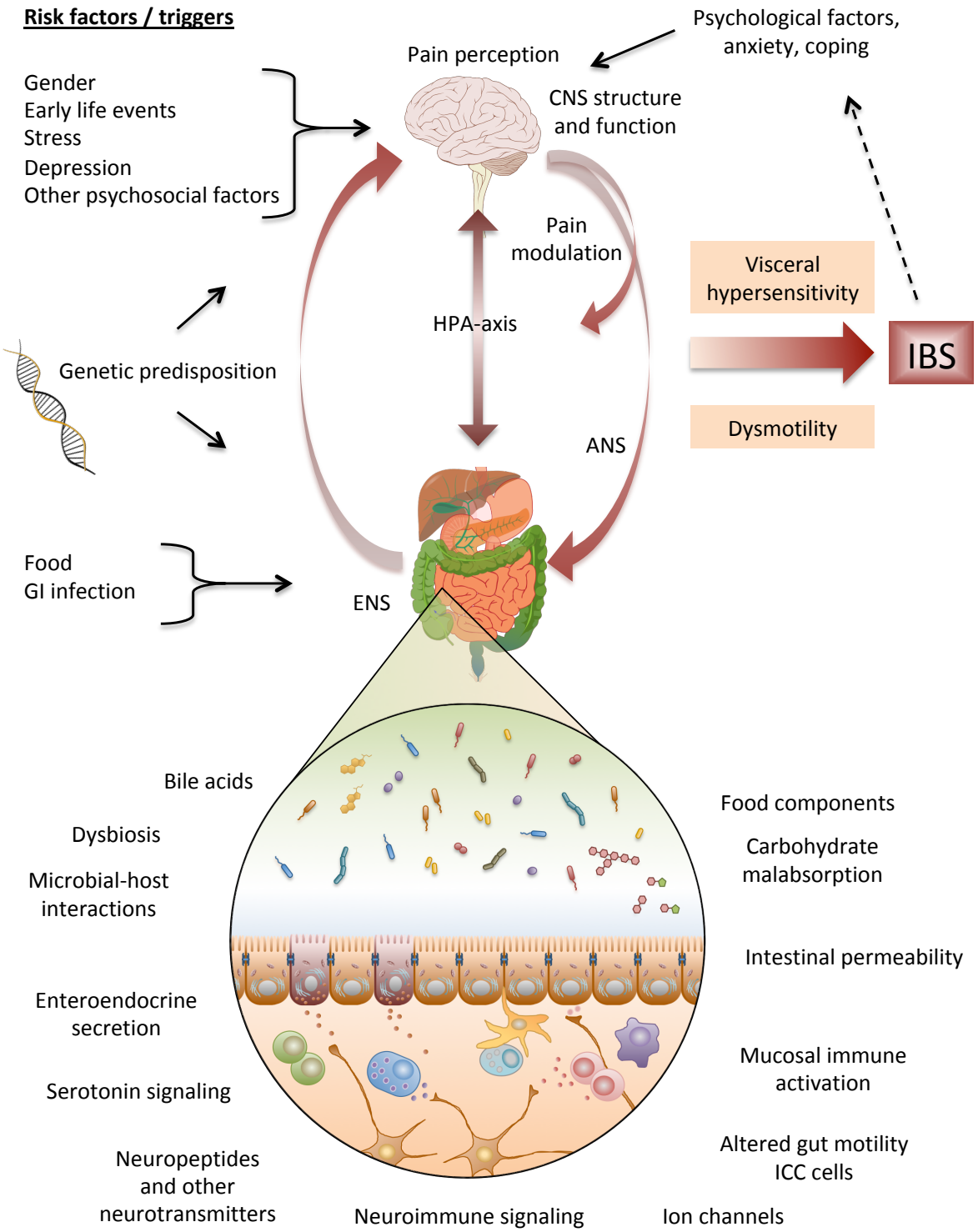


FIGURE 3. An overview of IBS pathophysiology. Several risk factors, triggers and other contributing or involved factors have been described in IBS, and while the etiology is not well understood, the overview picture tells us that this is a multifactorial condition with a brain-gut or gut-brain communication that is somehow 'out of tune' —a *disorder of gut-brain interaction*. Notably, the IBS phenotype is heterogeneous and key pathophysiological factors may differ between patients. At the same time, subsets of patients may have specific underlying abnormalities responsible for their IBS symptoms. ANS, autonomic nervous system; CNS, central nervous system; ENS, enteric nervous system; GI, gastrointestinal; HPA, hypothalamic-pituitary-adrenal; ICC, interstitial cells of Cajal.
© See page 71.

Gut microbiota is an extremely complex ecosystem residing in our GI tract, mostly in the colon, and consists of bacteria, archea, eukaryotes and viruses, although the bacteria domain by far outnumbers the rest. The rapid advancement in culture-independent molecular techniques (e.g., 16S rRNA sequencing) has enabled advancements in microbiota research and has shed light on its impact on various processes in the human body, with the microbiome now considered part of the gut-brain axis.[50, 85] Available studies on an altered microbial community in IBS compared to healthy individuals provide good evidence to conclude that there is a difference, at least in a subset of patients.[86, 87] Some studies suggest that while a subgroup of IBS tends to have a fecal microbial profile similar to healthy individuals, the other group shows a significantly different microbial pattern.[88, 89] At the phylum level, higher relative abundance of *Firmicutes* and lower relative abundance of *Bacteroidetes* (the two most dominant phyla in the gut) seem to characterize this difference.[87-90] However, there is heterogeneity and low reproducibility between studies, and it is not yet established exactly what types of microbial differences that exist and the causal effect in IBS is unproven.[85-87] Moreover, the proposed role of small intestinal bacterial overgrowth (SIBO) in IBS pathogenesis is controversial and still remains under debate.[86, 91]

The role of microbiota in IBS is very much unclear, but host-microbial interactions that may cause or influence IBS symptoms include activation of mucosal immune response, increase in intestinal epithelial permeability, activation of nociceptive sensory pathways and effects on the enteric nervous system.[86, 87] Interestingly, Crouzet *et al.* demonstrated that hypersensitivity to colonic distension can be transferred from IBS patients through fecal microbiota to germ-free mice,[92] and microbiota composition has also been linked to abdominal pain in a general population sample,[93] supporting a role of microbiota in visceral hypersensitivity. Moreover, fermentation of undigested dietary components is an important function that plays a major role in IBS symptom generation in some individuals, and this will be further discussed below. Gut microbiota is believed to have a heritable component where host genetics influence the formation of its composition.[94] Despite its complexity and high inter-individual variability, over time, the composition within one person is fairly stable.[95] However, various factors can influence the composition: dietary changes being the most obvious one.[96] Notably, a complex interplay exists between food, microbiota and intestinal transit time, as demonstrated in, for example, studies of germ-free and humanized mice,[97] and association studies showing correlation between fecal microbiota composition and surrogate measures (stool consistency and frequency) for intestinal transit time in population-based populations.[98]

Food components. The vast majority of IBS sufferers believe certain foods (including cereal-based, diary products, legumes, fatty foods, and certain fruits and vegetables) provoke or worsen their gastrointestinal symptoms, and postprandial (occurring after a meal) symptoms are common.[99-101] Although classical food allergies (IgE-mediated) are not convincingly linked to IBS, many patients still report on perceived food intolerances,[102, 103] and restrict their diet by avoiding foods perceived as problematic.[99, 104] A gluten-free diet has increased in popularity, not only in the IBS community but also in the general population, and the newly

defined condition non-coeliac gluten sensitivity (NCGS), experienced as intolerance to wheat products, is widely debated, as it is not clear whether the culprit is actually gluten.[105] Wheat contains also other compounds such as amylase-trypsin inhibitors, wheat-germ agglutinin, starch (main part of the endosperm), as well as dietary fiber.[104] The interplay between ingested food, products of digestion and the gut microbiota, is important to bear in mind when discussing IBS and diet, especially regarding carbohydrate and fiber intake. In a normal state (**figure 4**), the digestion of carbohydrates is initiated by salivary and pancreatic α-amylase, which hydrolyzes starch —the major carbohydrate component in our diet— into smaller glucose disaccharides (maltase, isomaltase) and oligosaccharides (e.g. maltotriose and branched α-limit dextrins). In the small intestine, the digestion of these starch products is finalized by α-glycosidases (brush-border enzymes), namely maltase-glucoamylase (MGAM), which hydrolyzes α-1,4-glycosidic bonds in maltose and maltotriose, and sucrase-isomaltase (SI), which hydrolyzes α-1,4-glycosidic bonds similar to MGAM but also branching α-1,6-glycosidic bonds in isomaltose and α-limit dextrins.[106] Sucrase is also responsible for the breakdown of sucrose, and lactase hydrolyzes lactose. The final products, monosaccharides, are readily absorbed by enterocytes into the blood stream. In the colon, gut microbiota ferment nutrients that escape absorption in the small intestine, such as fibers, resistant starch and other non-absorbed carbohydrates, producing short-chain fatty acids (SCFAs) and gases. Although normal and unproblematic for most people, some individuals will experience troublesome symptoms such as bloating, excessive gas, diarrhea and pain.[104] In this aspect, some of the major factors that may play a role in IBS and should be considered are *1) hypersensitivity to 'normal' luminal stimuli, 2) the amount of poorly and non-absorbed carbohydrates in the diet, and 3) the ability to digest and absorb generally digestible carbohydrates.*

The ingestion of food will always set off a cascade of responses throughout the GI tract.[107, 108] Different stimuli, such as luminal distension, osmolarity, or food components will trigger autonomic nerve signals and hormone release, which stimulate enteric neurons, smooth muscle cells, or endocrine and secretory cells, resulting in gut motor and secretory responses, as well as the transmitting of sensory information up to the CNS. The working hypothesis in IBS is that, for some reason, hypersensitivity to 'normal' visceral stimuli exists,[109] and it is therefore easy to assume that food must play a role, not necessarily as the underlying cause of IBS but at least as a trigger of symptoms in susceptible individuals prone to experience an exaggerated response to these stimuli.[1, 87] A potential role of fat or other food components has been suggested, but limited evidence exists and most of the latest research has focused on the role of poorly absorbed carbohydrates in IBS symptom generation.[104]

A generalized approach targeting several poorly or non-absorbed carbohydrates in the diet was developed in 2004 by Prof. Gibson *et al.* at the Monash University in Australia; the lowFODMAP (Fermentable Oligo-, Di- and Monosaccharides And Polyols) diet.[110] This diet is well recognized across the world and is already implemented in clinical practice. The evidence for its efficiency has increased lately, based mainly on randomized trials, and overall 50–80% of patients seem to benefit.[111] A randomized controlled trial (RCT) by

Halmos *et al.* observed a significantly lower overall GI symptom score in IBS patients on a lowFODMAP diet compared to a typical Australian diet, whereas no difference was seen in healthy controls.[112] Other RCTs have reported overall improvement (adequate relief) of IBS symptoms in more than half of patients going on a lowFODMAP diet, but notably, compared with a diet based upon modified National Institute for Health and Care Excellence (NICE) guidelines (instructions to eat small frequent meals, avoid trigger foods, avoid excess alcohol and caffeine),[113] or traditional dietary advice for IBS patients (limit the intake of insoluble fibers, fat, caffeine, gas-producing foods [i.e., legumes, onions, cabbage] and focus on regular and not too large-sized meals),[114] the effects were somewhat similar. Both types of

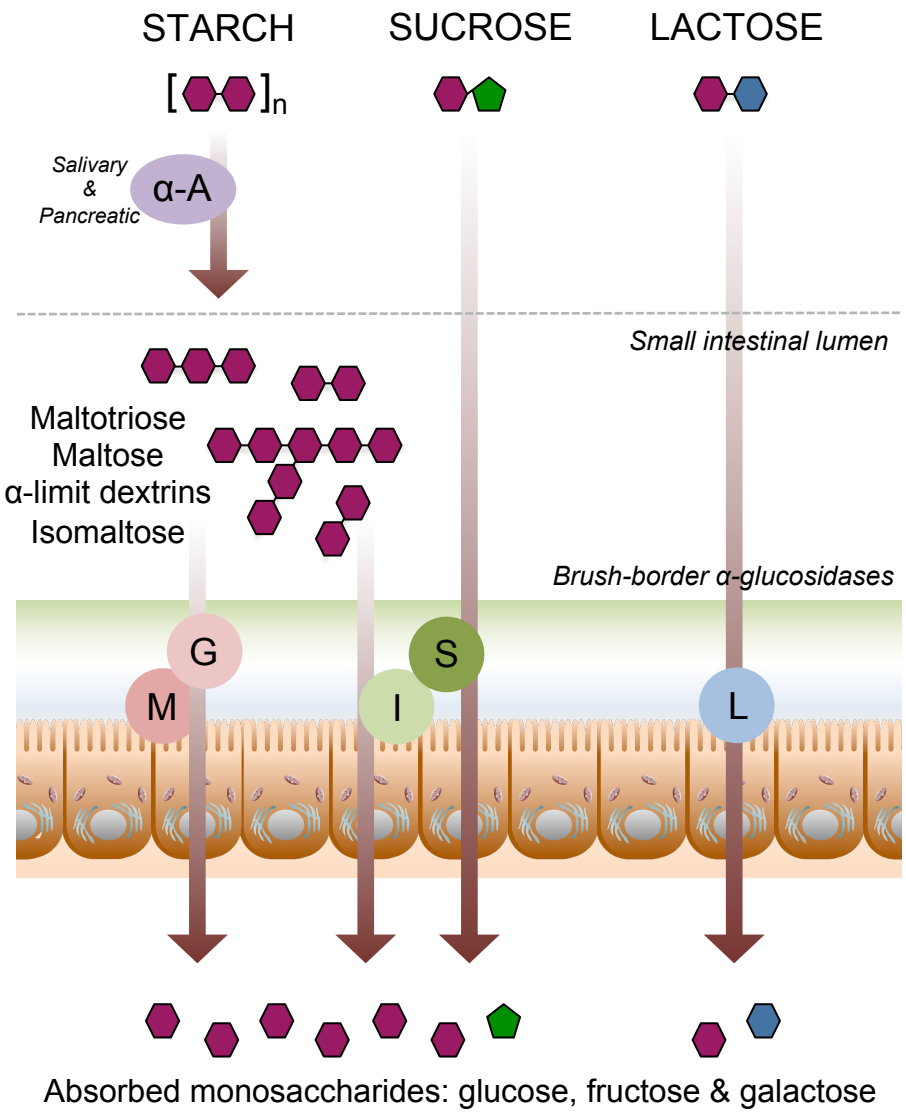


FIGURE 4. Human digestion of dietary carbohydrates in a normal state. Starch (the main carbohydrate component in our diet) is initially hydrolyzed by α-amylase from the salivary glands and pancreas, and finally broken down into glucose in the small intestinal brush border by maltase-glucoamylase and sucrase-isomaltase. Isomaltase is also able to hydrolyze the branching points of starch products, such as isomaltose and α-limit dextrins. Sucrose is hydrolyzed by sucrase, and lactose by lactase. The final products, monosaccharides, are readily absorbed through enterocytes into the blood stream. α-A, α-amylase; M, maltase; G, glucoamylase; I, isomaltase; S, sucrase; L, lactase.

intervention diets improved overall IBS symptoms, although the study that compared the lowFODMAP diet with a modified NICE diet could observe significantly greater benefits in abdominal pain and bloating scores in patients one the lowFODMAP diet.[113] The lowFODMAP diet is a 'top-down' approach, i.e., a strict elimination phase of a few weeks supervised by a dietician, is followed by a structured re-introduction of foods. This is important because of the potentially negative long-term effects on microbiota composition, quality of life, and the risk of inadequate nutrient intake.[115]

Poorly digested or malabsorbed carbohydrates (whether FODMAPs or other) accumulate in the intestinal lumen where they will increase osmolarity (which drives water retention) and undergo rapid fermentation by colonic bacteria (producing SCFAs and gases). This causes distension and bloating, activation of sensory neurons, and acceleration of transit time.[116] This is also the main mechanism in enzyme deficiencies, such as lactose intolerance, where undigested lactose accumulates in the gut and gives rise to symptoms such as diarrhea, abdominal pain and bloating.[117] In a survey from 2007, more than 50% of 1,242 interviewed IBS patients chose "Lack of digestive enzymes", among other pre-formulated answers, when asked to guess what causes IBS, and the responders were also most interested in knowing "What foods I should avoid".[118] Of note, treatment with pancrealipase, a combination of three digestive enzymes, has shown symptom improvement in postprandial IBS-D patients.[119] Moreover, in a study from 2012, low activity of one or more disaccharidases was detected in 50% of 44 children with dyspeptic symptoms,[120] and similar findings were reported in a recent study from 2017, where low disaccharidase activity was detected in 50% of 203 pediatric patients with normal histology who had undergone endoscopy with chronic abdominal pain as the primary indication.[121] In the clinical evaluation of suspected IBS patients, lactose and sometimes fructose intolerance (examined by hydrogen breath test or a trial period of elimination from the diet) may be considered.[1, 30] However, more rare enzyme deficiencies tend to be overlooked and may be misdiagnosed as IBS, in particular postprandial IBS-D.[101] Sucrase-isomaltase (SI) deficiency is an example of such a condition, and in **paper III**, through a series of experiments and association analyses of *SI* genetic variation, we provide evidence of a role of SI deficiency in IBS.

Moreover, ***Bile acids*** (BAs) are important for human digestion as they act as lipid detergents, enabling degradation and absorption of fat in the small intestine. The BAs are synthesized in the liver and secreted into the lumen of the duodenum, and after aiding in fat absorption, 95% are actively reabsorbed in the terminal ileum and recycled through the 'enterohepatic circulation of BAs'.[122] In the case of bile acid malabsorption (BAM), excess concentration of BAs in the colon may cause secretion of water and electrolytes, accelerate colonic transit and motility, alter mucosal permeability and increase visceral sensation, which together result in diarrhea and symptoms typical for chronic diarrhea or IBS-D.[51, 122] In fact, on average around 30% of patients with severe diarrhea-predominant IBS have evidence of BAM, as summarized in systematic reviews.[54, 123] Several plausible mechanisms of BAM have been proposed, with strongest evidence for a dysfunction in the negative feedback regulation of BA synthesis, a mechanism mediated by the endocrine fibroblast growth factor 19

(FGF19).[122, 124] Hence, bile acid malabsoption may be mainly responsible for symptoms in a particular subset of IBS-D.

1.3 ENDOPHENOTYPES OF IBS

As described above, IBS is usually sub-grouped based on predominant GI symptoms. However, over time, patients often move between IBS subgroups or even similar FBDs (**figure 1B**, page 2), indicating a spectrum of disease and not distinct entities.[11, 17-20] Considering this, the Rome criteria and symptom-based subgrouping may represent valuable instruments in the clinical management of IBS patients to help direct treatments (which often focus on symptom amelioration), but may be less informative in research and poorly applicable to predict the molecular mechanisms that contribute to IBS. Therefore, another alternative strategy for IBS gene discovery is to, in addition to the classical clinical phenotype, also use endophenotypes.[4] This concept can be traced back to 1966[125] and was introduced in the psychiatric field by Gottesman and Shields in 1973[126] as a way of deconstructing psychiatric illnesses into simpler components and to bridge the gap between symptom presentation and genetic variability.[127] Endophenotypes are measurable, often quantitative, intermediate traits, associated with both the disease and the underlying genetics, and have been widely used successfully not only in the psychiatric field but also for a variety of other complex traits, all of which include a genetic component and also constitute a group of conditions presenting with similar symptoms but with a potential mix of different underlying etiologies. Since endophenotypes typically are quantitative traits, an advantage from a statistical perspective to use them is also that they generally have better power to detect a genetic effect compared to binary traits.[128] Examples of endophenotype-disease relationships are blood pressure and cardiovascular disease, and bone mineral density and osteoporosis. By definition, the difference between endophenotype and similar terms, such as intermediate phenotype, biological marker or subclinical trait, is the genetic connection.[127]

In IBS, the most commonly used intermediate phenotypes (and potential endophenotypes) include measures of intestinal transit time and motility, visceral hypersensitivity and pain perception.[4, 129] Altered gut motility and visceral hypersensitivity have been known to be key elements in IBS for a long time.[10] The purpose of GI motility is to handle gut content, i.e., mix, store, and importantly, accomplish propulsion of gut content along the ~7 m long GI tract by peristalsis.[108] Intestinal dysmotility is common in IBS, and in those with abnormal transit time, slower transit time associates with constipation whereas faster transit time often indicates diarrhea,[130] hence intestinal transit time is considered a solid and objective trait for the evaluation of GI dysfunction. However, available methods and techniques to measure transit time require specific equipment and may be expensive and sometimes invasive, hence, and especially for the purpose of larger population-based studies, surrogate markers may be used instead. Stool consistency, assessed by using a Bristol stool form scale (BSFS), has shown to correlate well with transit time,[131, 132] and the BSFS tool is also used to characterize IBS patients according to the Rome criteria.[11] In addition, stool frequency (recorded through

bowel habit diaries) has also shown correlation and can therefore be considered another reliable surrogate marker for transit time.[130, 133]

Visceral hypersensitivity is central in IBS, and some studies have reported GI symptom severity to associate with visceral sensitivity.[134] Other studies have shown that about half of IBS patients are hypersensitive to visceral stimuli while the other half can be considered normosensitive,[83, 135] although the definite number is uncertain (various studies have described visceral hypersensitivity in 20–90% of patients).[109] Visceral hypersensitivity can be assessed using for example rectal barostat studies with recordings of rectal compliance (capacity of the gut to adapt to intraluminal distension),[108] and subjective measurements of the perception of visceral stimuli (i.e., pain and sensation thresholds and ratings).[77] Moreover, other potentially useful intermediate phenotypes, and proposed endophenotypes, in IBS include inflammatory markers (mucosal immune cells or inflammatory mediators), bile acid kinetics, intestinal permeability, or even gut microbiota composition (e.g., *Firmicutes/Bacteroidetes* ratio).[4, 129] Interestingly, recent studies have also explored the use of different brain imaging techniques to measure brain response to visceral stimuli in IBS, and in an interesting review of a systems view of IBS, Mayer *et al.* suggest that different brain signatures, or 'intermediate brain phenotypes', interact with intermediate gut phenotypes to together shape the clinical presentation of IBS.[52]

1.4 MANAGEMENT AND THERAPEUTIC OPTIONS

Because of its complexity, heterogeneity and largely unknown etiology, IBS is difficult to treat. There is no cure, and by using a trial-and-error approach, therapeutic management can only be based on patients' individual symptomatology with available treatment options directed towards symptom amelioration. Predominant symptoms, the level of severity, and patients' and practitioners' preferences, usually is what determines the choice of treatment.[30] Much of the pharmacological agents used in IBS focus on normalizing bowel habits, while visceral sensation and pain seem to be more difficult to treat.[136] Available pharmacotherapies include intestinal prosecretory agents (acting on ion channels or receptors on enterocytes, causing increased secretion of electrolytes and water into the lumen), antispasmodic drugs (agents that block muscarinic receptors or calcium channels on GI smooth muscle cells, causing relaxation and relief of pain), antidepressants (central sensory modulators, e.g., selective serotonin re-uptake inhibitors (SSRIs)), serotonin receptor antagonists/agonists (regulating gut motility and transit time), and others.[136, 137] However, most of these drugs have only low or moderate quality of evidence and their level of recommendation is often low.[136, 137] It is important though, to remember that IBS is a heterogeneous condition, and as long as IBS patients are all clustered together and sub-grouped solely based on symptoms, one cannot expect all IBS patients in a clinical trial to benefit from a specific treatment, and systematic reviews of their overall efficiency may be difficult to interpret. In the end, therapy strategies will be highly individual and, as long as a specific targetable cause is not identified in a patient, a combination of different types of treatments may be necessary, including also for instance dietary interventions, probiotics and psychological therapies.[30]

"Let food be thy medicine and medicine be thy food." Hippocrates

The first-line dietary treatment for normalizing bowel habits is to adjust the intake of fiber.[29] However, this is not as easy as it may sound, as a wide variety of dietary fibers exist and their properties (soluble/insoluble, viscous (gel-forming)/non-viscous, short-/long-chain, and their level of fermentation by gut bacteria) will determine the effects on IBS symptoms.[138] Supplementation of psyllium, a long-chain, soluble and moderately fermentable dietary fiber, has been especially recognized as beneficial for IBS global symptoms, with solid evidence for its efficiency,[137] whereas insoluble fiber (e.g., bran) does not seem to be of particular benefit in IBS.[137-140] However, available studies typically include all IBS in the same treatment group, hence data on subtype specific effects are lacking. Of note, increased fiber intake can also induce uncomfortable adverse effects such as abdominal pain, diarrhea or constipation, and bloating, and for this reason, their use in IBS is controversial.[138] Another dietary strategy, especially for diarrhea and bloating symptoms, is to try minimizing the production of gas by avoiding highly fermentable carbohydrates. This is the concept of the lowFODMAP diet (described in section **1.2.3** above), which limits the intake of a number of poorly absorbed short-chain carbohydrates due to their gas producing (through microbial fermentation) but also osmotic (attracts water) effects that can induce symptoms in some people.[111] The proportion of patients reporting symptom relief on a lowFODMAP diet is high (50–80%),[111] and can be compared with the clinical effect of certain drugs commonly used in IBS, such as Lubiprostone (a prosecretory agent, which activates ion channels in the intestinal lumen and is used for the treatment of IBS-C and chronic constipation) with 18% of patients reporting improvement in global symptoms.[141] However, some FODMAPs (fructo- and galactooligosaccharides) also have prebiotic effects, hence a lowFODMAP diet may have negative impact on microbiota composition,[142] and long-term effects are unknown.[111] Therefore, it is also notable, that when comparing the lowFODMAP diet with traditional dietary advice for IBS (NICE guidelines),[143] which is less restrictive and focus more on how and when to eat rather than on what foods to avoid, no significant difference for overall IBS symptoms is observed between the diets,[113, 114] —both dietary interventions are beneficial for about half of patients. This suggests that even though a strict diet can prove beneficial in IBS, it may not be necessary to introduce it to all patients. Instead, for some of them, smaller changes in dietary habits may be sufficient to improve their IBS symptoms, and dietary recommendation and guidance should be as individualized as possible.

Gut microbiota is an important player in IBS, acting as a link between several involved factors, and while providing health benefits for the host it can also contribute to gut symptoms. Thanks to its ability to adapt (by altering composition and/or activity) in response to a change in the luminal environment, gut microbiota also represents a promising therapeutic target. It can be modified using diet, or more specifically, prebiotics (non-absorbed fermentable carbohydrates, primarily oligosaccharides), but also with probiotics (live microorganisms with health benefits for the host), and even antibiotics. Available data has demonstrated that probiotics are effective in the therapy of IBS, not only in terms of improvement in overall IBS symptoms, but also specifically for abdominal pain, bloating and

flatulence (gas),[144] although it is unclear what types of bacterial strains should be used. Due to lack of data and heterogeneity between studies, there is insufficient evidence to conclude a positive effect from prebiotic or synbiotics (pre- and probiotics combined) specifically for the treatment of IBS.[144] Moreover, the antibiotic rifaxamin has shown efficiency for IBS-D in a few clinical trials, but the mechanisms are not clear and there are obviously concerns for long-term or repetitive use of antibiotics.[137]

Not to forget, IBS is in most patients a complex condition, and for successful clinical management, one must look at the big picture of the condition and address also extra-intestinal manifestations such as psychiatric comorbidities. There are a number of different therapies available that may be of benefit in the management of IBS, including cognitive behavioral therapy, hypnotherapy and other psychological therapies.[1, 137] Importantly, in addition to stress management and keeping in general a healthy diet, other lifestyle factors may be of importance. For instance, increased physical activity has shown to improve GI symptoms in IBS.[145] In summary, the clinical management of IBS may have to combine several lines of treatments, targeting the symptomatology from different angles simultaneously. At the same time, by gaining knowledge on the underlying pathophysiology and identifying useful biomarkers, we strive to improve the ability to better predict who is going to respond to what treatment.

1.5 GENETICS OF IBS

1.5.1 Studying the genetics of complex diseases; *what, when, why, how?*

***What** do we study?* In terms of our DNA, humans are approximately 99.9% similar, which, importantly, also means we are 0.1% different (or more, depending on what types of genetic variation is considered). This difference can be anything from large structural changes of the DNA sequence to changes in single base pair positions, i.e., single nucleotide variations (SNV), which is by far the most abundant type of genetic variation.[146] By definition, a common variant or single nucleotide polymorphism (SNP) is present in more than 1% of the population (minor allele frequency, MAF>0.01), whereas rare variants (often referred to as mutations) are less common than that. While most of these variants have negligible or unknown effects, some will for example cause an amino acid change in the corresponding protein (missense/coding variants) or may alter levels of transcription (expression quantitative trait loci; eQTL), which will have implications on biological systems in the body.[128]

'Genetic architecture' is a fancy term used to describe the characteristics of the genetic or heritable component of a phenotype/trait. It encompasses the type and number of genetic variations that influence the phenotype, their population frequencies, magnitudes of effects, as well as interactions with each other and the environment.[146] Rare genetic conditions, such as cystic fibrosis, are highly heritable and typically show a Mendelian inheritance pattern.[128] These conditions are caused by one or more rare variants in single genes, which have detrimental effects on the corresponding protein and strong influence (high penetrance) on the disease risk. However, most common diseases and traits, such as cardiovascular disease, diabetes or height, do not follow a classical Mendelian inheritance pattern even though they show heritability. These all have a complex genetic architecture with multiple genetic variants involved (i.e., polygenic). The 'Common Disease, Common Variant (CDCV)' hypothesis argues that the genetic component of common diseases is mainly composed of many variants that are also common in the population, all with relatively small effects (low penetrance) and risk ratios typically around 1.2–2.0.[128, 147] Indeed, GWAS efforts over the last few years have successfully identified common genetic risk variants for a variety of common diseases and traits. The National Human Genome Research Institute GWAS catalog (http://www.ebi.ac.uk/gwas/)[148] now lists over 58,000 unique SNP-trait associations from 3,308 publications (last data release 2018-02-21), indicating that the CDCV hypothesis is probably true for most common diseases. However, identified SNPs for a trait together often explain just a fraction of the genetic component, whereas a large portion remains unexplained.[128, 147] Take human height for example; in 2014, through large collaborative efforts and GWAS data from a total of 253,288 individuals, 697 genome-wide significant variants had been identified, though these variants together explain only 20% of the heritability for adult height.[149] Obviously, other forms of genetic variation or epigenetic effects may contribute to the 'missing heritability', but the 'Common Disease, Rare Variant (CDRV)' hypothesis argues that also rare variants can be the major contributor to common

diseases, and, as discussed by Schork *et al.,*[147] the truth probably lies somewhere in between. The genetic architecture can be a mix of both common and rare variants on a spectrum of different combinations for different traits,[146, 147] and IBS is not an exception.[4-6] A heritable component of IBS has been demonstrated (see next section **1.5.2**), and for most IBS patients, a complex and polygenic background with many genetic variants (mostly common) is thought to together contribute to disease (**figure 5**). Furthermore, specific combinations of these genotypes (together with environmental factors) may explain in part the clinical heterogeneity of the IBS phenotype, i.e., there are likely different sets of genes that can explain different features of IBS (e.g., related to diarrhea/constipation, pain perception, immune response or susceptibility to psychological comorbidity).[6] At the same time, evidence points to the existence of smaller IBS subgroups where rare high-penetrating single gene variants may principally account for their symptoms, as for example in the case of specific ion channelopathies (see **1.5.5** below).[5, 56]

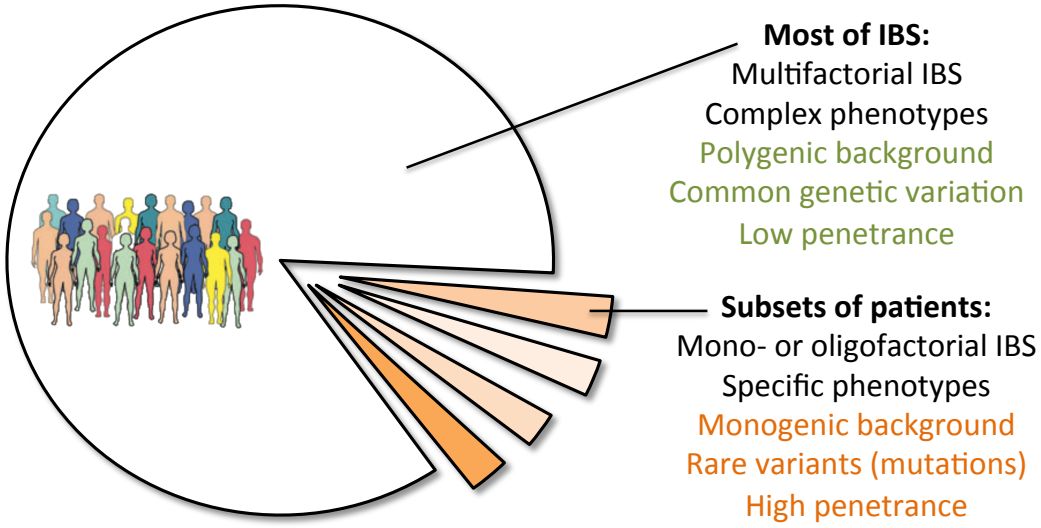


Figure 5. Hypothesis of the genetic background of IBS.

When *is the right time to study the genetics of a disease?* Whenever a trait of interest, for example IBS, shows a pattern of inheritance, as assessed by family aggregation or twin studies, then genetic studies can prove to be meaningful. Since the Human Genome Project, which successfully completed the first sequencing of the entire human genome in 2003,[150] technical as well as methodological development has expanded extremely rapidly, and is now in a very advanced stage compared to just a decade ago.[151] Especially the development of SNP arrays, containing broadly between 200,000 to several million SNPs on one chip (representing common variations across the entire genome), has enabled hypothesis-free studies on the genetic background of complex diseases through genome-wide association studies (GWAS). The considerable decreases in costs for these SNP arrays, together with sharing of genetic data (publicly available databases, and international collaborative efforts) in the gene-mapping community, are key factors that now enable large-scale analyses to be performed. In addition, the generation and mapping of functional annotation data, also in

specific disease-related tissues, provided by large projects such as ENCODE[152] and GTEx,[153] as well as a variety of available tools for post-GWAS functional analyses are of great help when trying to make biological sense out of the genetic association findings. Hence, we currently are right in the middle of an exciting era of genetic studies.[151]

Why*?* The ultimate goal of studying the genetics of a disease is to gain insight into the underlying biology, hoping that a better understanding of the disease will help in prevention, diagnosis, prognosis and improved therapy.[128, 146, 151] Specific genetic testing can serve as a diagnostic tool for Mendelian single-gene diseases (e.g., cystic fibrosis), but may be poorly applicable on an individual level for the diagnosis of complex polygenic conditions. However, genetic information may be used on a population level to predict who is at risk of developing the disease, in order to stratify people and direct preventive interventions to those at higher risk. This may be of particular importance for life-threatening conditions such as cancers (offering earlier or more frequent screening programs) or conditions where preventive interventions or lifestyle recommendations may be of particular importance, e.g., cardiovascular disease or type 2 diabetes. In terms of IBS, genetic research represents a valuable tool that will, through the identification of risk genes and key biological pathways, provide important insight into the pathophysiology. In addition to improve understanding of what it is that we call IBS, a major goal is to be able to re-classify this condition into novel subtypes based on molecular patterns and an underlying biology rather than the clinical symptomatology.[3, 5] This is in line with the *precision medicine* approach, aiming to better individualize treatment and management of patients by taking into account different layers of information and individual variability, including genomics.[154] While precision medicine is becoming a reality in some areas (e.g., cancer), it is the aim for also other complex diseases and disorders, such as in the psychiatric field,[155] as well as in IBS.[3]

Even though SNPs may have small effects on disease risk, their corresponding proteins or pathways may still be suitable targets for therapies and drug development.[146] One can argue that if small effects on protein level correspond to small effects on the disease, then if consistent, large effects on protein level may translate into large effects on the disease.[146] In other words, SNPs can help to highlight targetable proteins. There are many examples of pharmaceuticals that have proven clinically beneficial even though associated SNPs near the drug target each have small effects.[146] It is also possible that several associated SNPs or risk genes all act in the same pathway or process in the body. Hence, those findings may together point out a pathways relevant to the disease, which may be worth further investigation.

Moreover, the area of pharmacogenetics is emerging, which aims to understand how genetic variation influences drug metabolism (pharmacokinetics) or drug activity and receptor binding (pharmacodynamics), to be able to better predict drug responsiveness and proper dosing for different patients, in line with the movement towards precision medicine. Despite the lack of established risk genes, several pharmacogenetic studies in FGIDs have already been conducted, investigating the effects from genes in the CYP450, serotonergic,

cannabinoid, adrenergic and bile acid pathways.[156] This is an interesting area of research, although still in its infancy.

***How** do we study the genetic architecture?* For those who are not very familiar with genetic studies, here follows a short section on the basic principles and workflow for conducting such studies. Genetic studies test the relationship between genetic variation and a phenotype of interest, and while a well defined phenotype is the key in these studies, this is also the fundamental issue and challenge when it comes to IBS (see **1.5.4** below). Large international projects, such as the HapMap[157] and 1000Genomes[158] projects, have been crucial for enabling genetic studies in different populations, as these have sequenced a number of people from different populations and created publicly available catalogues of their genetic sequence. These serve as reference panels providing important information on 1) the location and density of genetic variants, 2) the relation between these (described as linkage disequilibrium [LD]; a measurement of co-inheritance based on recombination rates in a population) and their haplotype structures, and 3) differences between populations. These catalogues have been particularly useful for designing chip-based microarrays (for the purpose of GWAS), since one can pick suitable markers that act as proxies for related SNPs and thereby covering more of the variation across the genome without having to genotype redundant information. Closely associated SNPs (based on LD) can instead be imputed afterwards based on these tagging markers and linkage information in appropriate reference panels.[128] A limitation of many of these SNP arrays is that they cover well the common polymorphisms (MAF>1%) but poorly capture rare variants, but as technology is developing and costs go down, we are moving into an era where high coverage sequencing chips that include also rare variants (exome and whole-genome sequencing) are more frequently used.[151]

Once phenotype information has been collected, and genetic variation captured, each SNP can be tested independently for its association with the phenotype. In hypothesis-driven candidate-gene studies or replication studies for the validation of previous results, a few genotyped variants in a specific region may be tested. On the other hand, in a hypothesis-free GWAS approach, hundreds of thousands to millions of SNP-phenotype tests are simultaneously conducted across the entire genome. Obviously, with that many tests performed, there will be a need for multiple testing correction, and based on a simple Bonferroni correction, the gold standard in GWAS is currently a *p*-value threshold of 5.0×10^{-8} (α of 0.05 /1,000,000).[128, 159] To statistically test the association of a genotype with a binary trait (case/control), contingency table tests (Chi-square, Fisher's exact or Cochran-Armitage trend test) can be used or, if covariate adjustment is necessary, typically logistic regression. Moreover, there are a number of models that test different types of genetic effects (allelic, genotypic, dominant, recessive and other) but the additive genetic model, which assumes a linear increase in risk for each copy of the risk allele, is most commonly used as it has reasonable power to detect both dominant and additive effects.[128, 159] Covariates should be considered, such as gender or study center, and in GWAS also the potential impact of population stratification. Moreover, before association testing can be performed in a GWAS, rigorous quality control (QC) is performed on the data to check the quality of samples

(including call rate, gender check, unexpected relatedness and population stratification) and markers (genotype call rate, MAF and Hardy-Weinberg equilibrium).[160] Appropriate computational software's to use include, among others, R, SNPTEST and PLINK (publicly available), or the commercially available SNP & Variation Suite (SVS).

Importantly, results from genetic association studies should then be validated through replication in independent cohorts, and meta-analyses or pooled analyses may also be performed combining the results from several similar studies to evaluate if the same effect is seen throughout and to increase sample size and power to detect association.[128, 159] Finally, the challenge of interpreting the results and linking them to biology remains, which is not always that straightforward.[151] Various post-association analyses can be performed for fine-mapping of results, to gain information about the genomic content and try to identify causal variants responsible for the association. *In silico* analyses (using available online tools and databases) can also help in predicting the effects from risk variants on gene products, correlating with expression levels in different tissues, or perform pathway analyses through for example gene-set enrichment analysis (GSEA). Moreover, other functional characterization analyses include *in vitro* experiments and studies of mRNA or protein levels in biological material from patients and healthy controls.

1.5.2 Heritability of IBS

A heritable component in IBS is present, and has been demonstrated in several ways.[6] Some studies have evaluated the familial clustering of IBS by simply asking affected members if they have another family member affected by IBS (i.e., proxy reporting), but since the accuracy of this type of reporting is poor,[6, 161] Saito *et al.* performed a larger family case-control study in 2008.[162] By collecting IBS symptom data (questionnaires) and medical history (electronic medical register) for 477 IBS cases, 297 controls and their (1,492 and 936, respectively) first-degree relatives, they found it to be 2–3 times more likely that a relative of a case had IBS (50% of cases had at least one affected relative) compared to controls (27% had at least one affected relative). Several twins studies on IBS have estimated the heritability —the percentage of phenotypic variance that is due to inherited genetic factors— to range between 0–57%,[6] suggesting a genetic component despite the notable heterogeneity in heritability estimates. Moreover, in 2015, a comprehensive register-based Swedish national survey, including 51,952 individuals diagnosed with IBS, showed increased risk of IBS among first-, second- and third-degree relatives, clearly demonstrating the existence of a genetic component.[163] Not surprisingly, a non-genetic (environmental) component was also suggested to contribute, as seen by the clustering among spouses in this study. In addition, the same authors recently contributed with a study on familial aggregation of IBS among adoptees, and could confirm once again a role of genetics as the risk of IBS in adoptees was higher if their biological parents, rather than their adoptive parents, were diagnosed with IBS.[164] Heritability in this study was estimated to 19.5%±8.5%. Taken together, several lines of evidence suggest IBS has a substantial heritable component, but that genetic factors also interact with environmental factors to form this complex phenotype.

1.5.3 IBS genetics so far

Genetic studies, and especially GWASs, have been successful in mapping the genetic background of a variety of complex diseases, including gastrointestinal conditions such as inflammatory bowel disease (IBD) where a total of 200 genetic risk loci have been identified.[165, 166] However, IBS has been very much lagging behind.

In 2013, at the time of the start of this PhD project, more than 60 genes had been investigated for their potential role in IBS and its clinical subtypes. These have already been extensively reviewed elsewhere,[3, 6, 129, 167] but include genes involved in immune function, bile acid metabolism, intestinal barrier function, neurotransmitter signaling, nociception and others; i.e. covering most of the above mentioned mechanisms/pathways that seem to be of importance in IBS. These were all candidate-gene studies with a prior and plausible hypothesis of a putative role of a specific gene in IBS pathophysiology, for example its involvement in GI motor or sensory function, or the immune response to the intestinal milieu. It should be noted though, that all of these previous studies were conducted in relatively small sample sets with often conflicting results, and most of the time not successfully validated or replicated in independent cohorts; hence, overall we had very few convincing IBS risk genes.[4, 5] However, one exception might be *TNFSF15*, a gene encoding the TNF-like ligand 1A (TL1A), which is expressed by immune cells and amplifies the inflammatory response in the gut mucosa (**figure 6**).[4] The original study conducted in our lab and the Mayo Clinic,[168] aimed to investigate 30 established Crohn's disease risk loci for their potential association with IBS, and found the risk allele G of the *TNFSF15* SNP rs426839 to be more common in IBS cases than in controls (p=2.2×10^{-5}; OR 1.37), especially in IBS-C (p=8.7×10^{-7}; OR 1.79). The effect was observed in both of the case-control studies used (Sweden and USA, total n=1,992), and has also been replicated in additional cohorts since then.[169-171] In the original study, the risk allele was also associated with higher TNFSF15 mRNA levels in blood and rectal mucosa,[168] which, taken together, supports the hypothesis of immune activation in IBS, at least in a subset.

Also other immune related genes have been suggested to play a role in IBS, but reported results have been inconsistent and further studies are warranted.[171] Moreover, because of the presence and function of serotonin in both the brain and the gut, a large portion of the study efforts in IBS genetics so far has been put on the serotonergic system.[6, 172] In particular, genetic variation in the promoter region of the serotonin reuptake transporter (*SLC6A4*) has been associated with IBS-D and IBS-C, but also with IBS comorbidities (depression and anxiety).[3] Other genetic studies have focused on serotonin receptors and have provided some evidence of their involvement in IBS-D, especially the serotonin type 3 receptor (5-HT$_3$), which is an ion channel encoded by the *HTR3* genes. These studies together support the notion of disrupted serotonergic signaling in IBS, but further investigations are needed to confirm this hypothesis. Another gene worth mentioning is *CDC42* (cell division cycle 42). This encodes a GTPase protein involved in the control of epithelial barrier function and has been linked to IBS-C in two independent cohorts.[170] Nevertheless, at the time of initiation of

this PhD project, with possibly the *TNFSF15* gene being the only exception, there were basically no unequivocal risk genes identified for the clinical phenotype of IBS.

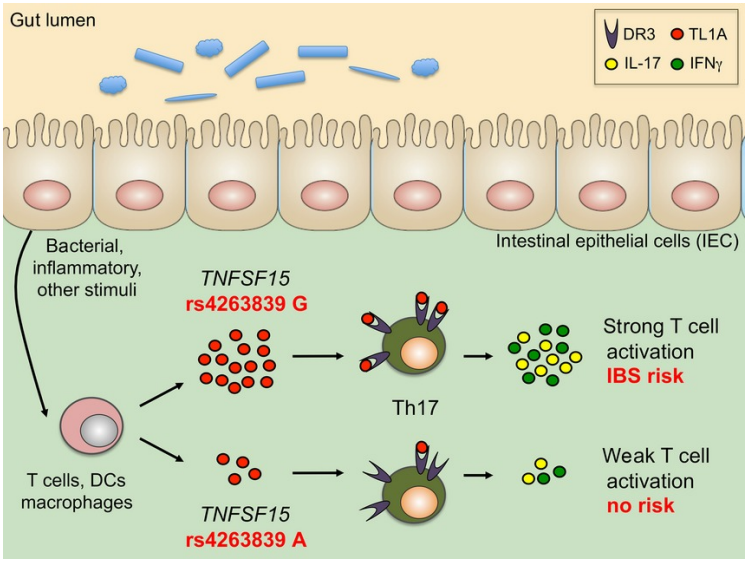


Figure 6 Schematic illustration of the hypothetical mechanism underlying association of *TNFSF15* polymorphism with IBS. DR3, death receptor 3; IFNγ, interferon gamma; Il-17, interleukin 17; TL1A, TNF-like ligand 1A. D'Amato,[4] © see page 71.

Another attractive approach for elucidating the genetic background of IBS and other FGIDs is the endophenotype approach, which I introduced in section **1.3** above. By using related intermediate traits instead of the typical clinical phenotype, the concept of this approach is to try reducing the complexity of the phenotype (disease) studied, and instead increase chances of finding genes important for the underlying biological pathways that contribute to the pathophysiology. Two convincing examples of this genotype-intermediate phenotype association in IBS are the neuropeptide S receptor (*NPSR1,* see next section) and the Klothoβ (*KLB)* gene, both of which have been implicated in lower GI motility.[77, 124] Klothoβ is a co-receptor with the fibroblast growth factor receptor 4 (FGFR4) on hepatocytes, and the coding SNP rs17618244 has been significantly associated with colonic transit time at 24 h in IBS-D (p=0.0018).[124] In the enterohepatic circulation of BAs, reabsorption in the ileum causes negative feedback on hepatic BA synthesis, and this is mediated by this FGFR4/ Klothoβ complex upon binding of the endocrine factor FGF19 at the cell surface of hepatocytes.[122] Using a protein stability assay in HEK293 cells, the KLB Arg728 variant that associated with increased colonic transit time also appeared to reduce protein stability, which can explain its functional significance.[124] An instable Klothoβ protein may limit the ability to down-regulate BA synthesis, which in turn causes increased concentrations of BAs in the intestines, resulting in accelerated transit time and bile acid induced diarrhea.[122] Hence, this is an example of a specific mechanism that may be responsible for symptoms in a subset of IBS-D patients.

Moreover, keeping in mind that IBS often arises from the interaction between genes and environment, and that psychosocial factors, stress, early life events and exposure to pathogens influence IBS risk, it is plausible to also hypothesize a role for epigenetics.[1, 3] A few studies

have reported altered expression profiles of microRNAs in IBS, in particular IBS-D, and among others, Zhou *et al*. could demonstrate a role of certain microRNAs in visceral hypersensitivity and suggested that decreased expression of miR-199 contributes to visceral pain through upregulation of the ion channel TRPV1.[173] Some animal studies also suggest that epigenetic mechanisms (such as DNA methylation or histone modification) may be implicated in stress-related alteration of the HPA-axis. However, epigenetic and microRNA studies are few, and these areas of research, as well as the area of pharmacogenetics, still remain in their infancy.[3, 156]

1.5.4 Challenges in studying the genetic architecture of IBS

'Gene-hunting' in IBS is extremely challenging.[3-6] First of all, a clearly definable and stable phenotype is the cornerstone of any genetic study, and already there we hit our first (and major) obstacle. A trait/disease becomes more challenging to study when there is considerable heterogeneity, and this relates both to the level of precision to which the trait can be measured or diagnosed, but also to the biology behind it.[151]

Measuring IBS is difficult. Due to our limited knowledge on the etiology, there are no established biomarkers or diagnostic tests,[2] and symptom-based criteria is the closest we have to a gold standard for diagnosis and sub-classification of IBS and other FGIDs, although, in clinical practice IBS is still often treated as a 'diagnosis of exclusion'.[31] In addition, although regular updates of the Rome criteria (revised three times since 1990) are necessary in order to adapt to the increased knowledge that gradually emerges in FGIDs, this also means the definition of IBS is not stable over time. Moreover, the biology behind IBS also appears to be heterogeneous, as IBS is believed to be a constellation of different disorders gathered under the same umbrella of similar symptoms. Of note, this will be reflected in the genetic background, where some genes may be specific for certain subgroups or intermediate phenotypes of IBS.[4, 5]

To compensate for this heterogeneity, large sample sizes will be necessary in order to improve statistical power of detecting an association. However, the number of available case-control sets with well-characterized IBS patients from the clinics is limited, and while these serve as ideal replication and validation cohorts, the sample size needed for the discovery of risk genes and variants is considerably larger, at least if GWA studies are to be carried out.[4] In addition, considering the heterogeneity of the IBS phenotype and genotype, when combining or comparing data from different cohorts there is a risk of introducing center-specific recruitment bias.[3, 4] Suppose, for example, that IBS would be either a 'brain-to-gut' (i.e., psychiatric or stress conditions arise first and then influence GI function) or 'gut-to-brain' (i.e., gut immune activation, ion channel dysfunction, or other factors that originate in the gut, drive the symptoms), then different focus and expertise at recruitment centers or the specific aims of the original investigation, may result in differences in the recruited study cohorts. In other words, a set of IBS cases from one center may be mostly characterized by psychiatric comorbidities whereas cases from another center perhaps originally sought medical attention due to suspected IBD or celiac disease, and might therefore be more prone

to have an inflammatory background. Hence, presumably, these groups would not only differ in terms of their etiology, but also in the genetic background with different sets of 'IBS risk genes' influencing functions primarily in the brain or the gut.[4]

Not to forget, collecting controls can also be a challenge. Ideally, controls should be recruited in the same way as cases, using the same investigative instruments for their classification.[3, 4] Considering the high prevalence of IBS in the general population (also those fulfilling Rome criteria although they haven't been diagnosed in the clinic), possibly hidden cases can be present among controls if IBS is not checked for. Moreover, exclusion criteria such as IBD or celiac disease should obviously also apply for both cases and controls. Finally, if a Rome module of questions is used, then individuals that fulfill only part of the IBS criteria should be classified as neither cases nor controls.[4]

1.5.5 Our approach and previous work

Recognizing that IBS appears to be a highly heterogeneous phenotype spanning from complex polygenic conditions to more rare single gene forms (**figure 5**, page 20), different strategies are needed for the identification of its genetic component.[4, 5] This implies hypotheses driven candidate-gene studies but also large-scale genome-wide efforts. It may also be useful to challenge the traditional clinical phenotype by using other definitions or intermediate phenotypes. Importantly, results coming from these studies should then be validated through replication in well-characterized IBS cases and controls from specialized clinics, using targeted genotyping. To investigate also rare variants/mutations in subgroups of patients, sequencing of specific genes in well-selected cases and controls is necessary, and eventually even next-generation sequencing efforts of the entire exome or genome (**figure 7**, page 30).

An excellent example of the existence of rare, but highly penetrant, single gene variants in subgroups of IBS, is the work done on *SCN5A*. This gene encodes the α-subunit of a voltage-gated sodium channel ($Na_V1.5$) responsible for the pacemaker function of the heart, and mutations in this gene are known to cause channel dysfunction (channelopathies) leading to cardiac arrhythmias, including Long QT and Brugada syndrome.[174] However, $Na_V1.5$ is also present on intestinal smooth muscle cells and the 'pacemaker' cells of the gut; the interstitial cells of Cajal (ICC),[175-177] which creates slow waves that serve as an underlying rhythm for gut motility. The interesting observation that cardiac arrhythmia patients with *SCN5A* mutations often present with IBS-like gut symptoms,[178] led to the hypothesis of a potential role of $Na_V1.5$ channelopathies in IBS. Following a pilot study, where a rare *SCN5A* mutation was found in one of 49 IBS patients,[179] the hypothesis was further investigated at the Mayo Clinic in collaboration with our group, where the *SCN5A* gene was sequenced in 584 IBS patients and 1,380 asymptomatic controls.[56] Indeed, missense mutations were found in 13 (2.2%) of patients but none of the controls. Most of these mutations had loss-of-function effects, and carriers most often had IBS-C. Of note, a severely constipated patient with the most highly penetrating *SCN5A* mutation identified could be successfully treated with a drug (mexiletine) known to rescue $Na_V1.5$ defects. In addition, an association signal of common

variants was detected for the *SCN5A* gene in our pilot GWAS of IBS (described below)[180] and by genotyping 17 SNPs from this locus in four additional independent case-control cohorts (total n=1,745), this association could be confirmed.[56] Hence, these findings not only support the notion of certain IBS subgroups having rare single gene conditions, but also that common variants may play a role (for instance through transcriptional control of the gene). Since ion channels are directly involved in both gut motility and visceral sensation, these represents ideal pathophysiological candidates and therapeutic targets in IBS.[80] Therefore, in a follow-up study (**paper II** in this thesis), we aimed to investigate the role of also other ion channel genes in IBS.

The traditional subgrouping of IBS, based solely on the presence of diarrhea and/or constipation (Rome criteria), can be a valuable instrument in the clinical management of patients as a help in choosing a treatment approach, but may not perform as well in predicting molecular mechanisms in research, or to identify specific genes that influence these mechanisms.[4] For that reason, an alternative strategy for IBS gene hunting is to also use intermediate phenotypes (as potential endophenotypes), such as colonic transit time or visceral sensation (e.g., pain thresholds or scores in response to visceral stimuli); a concept introduced in section **1.3** above (and mentioned in **1.5.3**). In line with this approach, our group has previously investigated the role of neuropeptide S (NPS) and its receptor NPSR1 in IBS-related intermediate phenotypes in a study of 699 cases with FGIDs and 233 healthy controls.[77] While no significant association could be seen for the seventeen genotyped *NPSR1* SNPs by comparing symptom-based phenotypes (IBS/IBS-D/IBS-C/dyspepsia etc or total FGIDs) with healthy controls, instead, association was shown between some of the *NPSR1* polymorphisms and colonic transit time as well as sensory rating (gas, urgency and pain) in response to barostat-induced visceral distention. As part of the brain-gut communication, NPS-NPSR1 signaling is implicated in the modulation of anxiety, stress, fear, inflammation and nociception,[181-186] and polymorphisms in the *NPSR1* gene have previously shown association with anxiety and depression,[183, 184] atopy and asthma,[187-190] as well as chronic inflammatory diseases such as IBD[191] and rheumatoid arthritis.[192] The NPSR1 receptor is expressed in the brain but also in enteroendocrine cells in the gut,[186] and previous work from our group have also demonstrated that NPS-NPSR1 signaling can induce the expression of other neuropeptides and gut hormones, including neurotensin, cholecystokinin, vasoactive intestinal peptide and somatostatin.[77, 186] Based on these findings, and the proposed role of this receptor in nociception, it was then hypothesized that NPSR1 may be involved in visceral sensation and pain, which led to **paper I** in this thesis, where *NPSR1* polymorphisms were tested for their association with recurrent abdominal pain (RAP) in children from a large Swedish birth cohort.

Another recent example of the use of intermediate phenotypes in IBS genetic research, is a study coming from our group in collaboration with A Zhernakova (University of Groningen); the first GWAS of stool frequency.[84] This study was conducted in two population-based cohorts from Sweden (Population-based Colonoscopy study; PopCol, n=284) and the Netherlands (LifeLines-DEEP, n=1,546).[84] Although no genome-wide association signal was

detected, plausible candidate genes were identified, including genes involved in the cytochrome P450 pathway of drug and xenobiotic metabolism. Intriguingly, a broader post-GWAS gene-set enrichment analysis of the genes located at the 53 loci showing suggestive association ($p < 5 \times 10^{-5}$) with stool frequency indicated enrichment of those genes in pathways related to ion channel activity. In another study with the same lead author (F Hadizadeh, recently graduated PhD from our group), stool frequency was shown to associate with gut microbiota composition in 69 individuals from the Swedish PopCol cohort.[98]

PopCol is a general population-based cohort of Swedish randomly selected individuals, of whom 745 volunteered to undergo an ileocolonoscopy, and a subset of these also kept detailed diaries of their bowel habits and symptoms for 7 and 14 days. In addition, blood and stool samples were collected.[193, 194] Hence, PopCol represents a data-rich resource that has been particularly useful to explore the results from our candidate-gene studies in a general-population sample, by for example correlating genotype with intermediate phenotypes, such as stool frequency or BSFS scores, or even abundance of fecal microbiota genera. In this thesis, data from PopCol has been exploited in both **paper II** and **III**. As mentioned above, the aim of **paper II** was to investigate the role of ion channel genes in IBS. **Paper III** is a nutrigenetic type of study where we through several steps tested sucrase-isomaltase (*SI*) genetic variants for their potential relevance in IBS, hypothesizing that SI deficiency (causing maldigestion and -absorption of sucrose and starch), although partial or in milder forms, is present in a subset of IBS patients.

As described in **1.5.3** above, a number of studies have previously been performed in IBS, trying to investigate the role of specific candidate genes based on plausible hypotheses of their involvement in IBS pathophysiology. However, most of these studies suffer from limitations (mainly small sample sets and lack of replication data), —perhaps with *TNFSF15*, described above, as the only exception—, and it became clear that in order to eventually identify true unequivocal IBS risk genes and variants, especially common variation, large-scaled analyses need to be implemented.[4] Hypothesis-free GWA studies, investigating the entire genome, have been successful in a plethora of other complex diseases,[151] but before the initiation of this PhD project, similar efforts had not been performed in IBS. In order to overcome some of the major challenges in IBS genetic research (including a heterogeneous phenotype and lack of suitably sized clinical cohorts) we proposed a few years ago, that a powerful approach might come from shifting to general-population samples (**figure 7**).[4, 5]

Large biobanks and epidemiological cohorts from the general population, although originally often designed to answer other research questions, offer great opportunities to study also IBS because of its high prevalence in the general population. Large amount of data has already been collected in these cohorts, and despite an obvious loss in specificity, utilization of these resources will provide a considerable gain in sample size. Only the 'tip of the iceberg' of IBS sufferers is found in the clinics; most of them are undiagnosed, although fulfilling the IBS Rome criteria.[37, 38] Hence, by using for example Rome criteria in questionnaires, we should be able to capture more of the IBS sufferers present in the general population. Depending on

what data is available, genotype data may also be linked to International Classification of Diseases (ICD) codes in electronic medical records (EMR), or even self-reported IBS (participants answering yes/no to *"Have you ever been diagnosed with…?"*). Reported diarrhea, constipation, abdominal pain, or intermediate phenotypes such as bowel movement frequency or Bristol stool form scale may also be exploited as alternative traits, depending on data available. Moreover, another advantage with working with general-population cohorts is that controls may be more appropriately defined, as they are co-sampled with the cases, and the very same investigative tools can be used to characterize both groups. For instance, considering the high prevalence of IBS symptoms, 10–20% of any general population sample may in fact constitute 'hidden' cases; hence, excluding individuals with reported GI symptoms from the control group should be helpful in avoiding these 'false controls'.

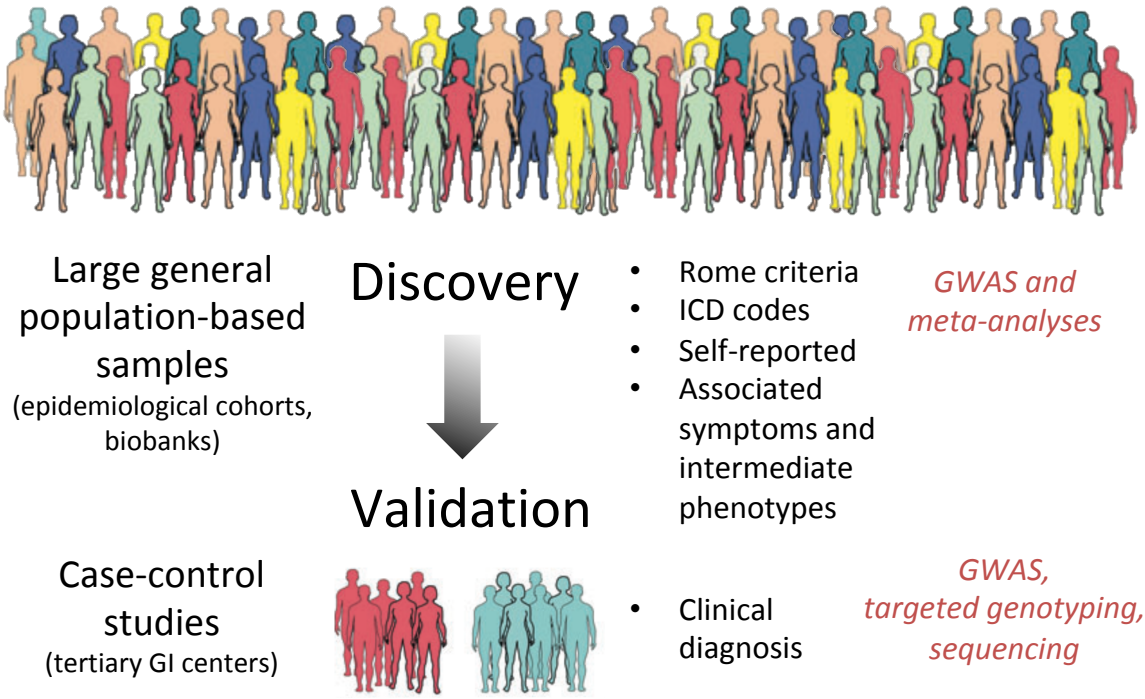


Figure 7. The proposed general population-based approach for the discovery of IBS risk genes and variants. Large general population-based samples may be exploited for IBS risk gene discovery efforts, and results coming from these studies then validated in well-characterized clinical IBS cases and controls. GI, gastrointestinal; GWAS, genome-wide association study.

Shortly after my registration as PhD student, our group published a first GWAS of IBS, which marked the takeoff of our general population approach.[180] In that study, a questionnaire module compatible with the Rome II criteria was used to identify 534 IBS cases and 4,932 asymptomatic controls from TwinGene, a subset of the Swedish Screening Across the Lifespan Twin study (SALT),[195, 196] where heritability of IBS has been previously estimated to be 25%.[197] Suggestive regions of association were detected in the GWAS and were further replicated in six independent clinical case-control cohorts from Europe and the US (replication total n=3,511) (**figure 8**). One of these loci showed consistent effect on IBS risk in all of those study populations (meta-analysis p=9.31×10^{-6}); a locus on chromosome 7

harboring the genes *KDELR2 (KDEL endoplasmatic reticulum protein retention receptor 2)* and *GRID2IP (glutamate receptor, ionotropic, delta 2 interacting protein)*. Even though no association signal reached genome-wide significance (total sample size of 8,977 likely still not big enough), this study served as a proof-of-principle pilot GWAS and corroborated our hypothesis that there lies a great potential in utilizing large general population-based cohorts with available genotype and healthcare/epidemiological data for the purpose of discovering IBS risk genes.[180]

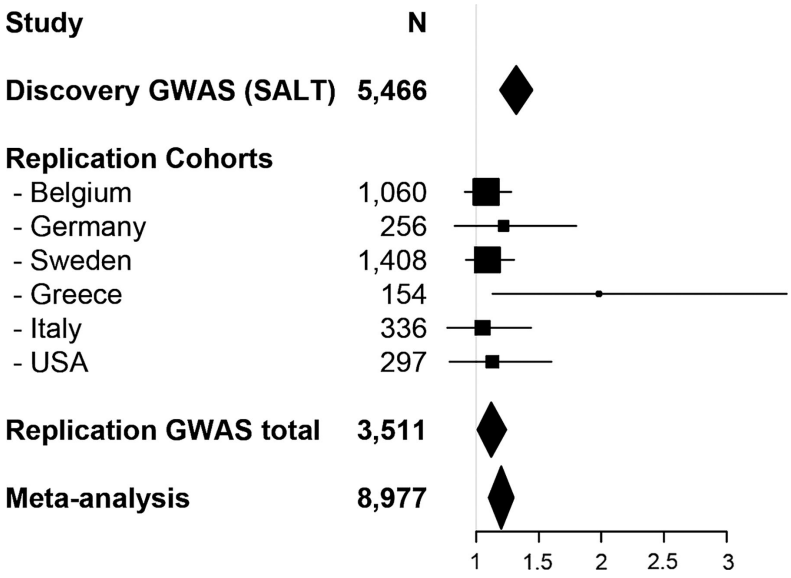


Figure 8. Forest plot showing the association between IBS and the lead SNP (rs12702514) of a suggested IBS risk locus on chromosome 7. Individual and cumulative ORs and 95% confidence interval are shown together with sample size for the discovery cohort TwinGene (part of SALT), each replication cohort, and the total study cohort. GWAS, genome-wide association study; OR, odds ratio; SALT, Screening Across the Lifespan Twin study. Ek *et al.,*[180] © see page 71.

In **paper IV**, we continued on this approach by combining five independent GWA studies from cohorts around Europe (namely LifeLines-DEEP, SHIP-Trend, TwinsUK, the Northern Finland Birth Cohort 1966, and TwinGene) and combined the summary statistics from these GWASs in a large meta-analysis involving a total of 1,335 IBS cases and 9,768 controls.

Excitingly, this way of 'thinking outside the box' has grown into a large collaborative ongoing project named *the bellygenes initiative* (www.mdalab.org/bellygenes), which is led by PI Mauro D'Amato and co-PI Alexandra Zhernakova. Through international collaboration, this project has received increased interest, and finally a large number of general (European population-based cohorts) and dedicated (IBS case-control samples from expert clinics in Europe and the US) resources have been gathered that enable studies of IBS genetic architecture with unprecedented potential.

2 AIMS OF THE THESIS

2.1 OVERARCHING HYPOTHESES

Based on available and recent evidence in the IBS research field, we believe that:

- IBS is a constellation of disorders with similar symptoms but possibly different underlying pathophysiological mechanisms.
- Genetic predisposition contributes to IBS.
- The genetic background for most IBS patients is complex and polygenic with many genetic variants together contributing to disease.
- Smaller subgroups of IBS exist where rare highly penetrating single gene variants may principally account for their GI symptoms.

2.2 AIMS

The overall aim of this thesis was to identify, validate and functionally characterize genetic factors predisposing to IBS and its associated gastrointestinal symptoms and intermediate phenotypes. However, as IBS genetic research is still in its infancy due to a complex and heterogeneous phenotype that is extremely challenging to study, the scope of the thesis has mainly focused on the *discovery* of IBS risk genes and variants. The ultimate goal with our research is to identify key pathophysiological mechanisms that can help explain the etiology of IBS and improve diagnosis and the classification of disease subtypes, eventually leading to the delineation of novel and personalized therapeutic options. We strive to do so by adopting different methodological approaches, with the following specific aims:

- Candidate-gene studies, with a hypothesis-driven approach to evaluate the role of a specific gene or set of genes that may play a role in IBS pathophysiology (**paper I, II** and **III**).
- Large-scale analyses, i.e., genome-wide association studies (GWAS) and their meta-analyses to capture common, low-penetrance gene variants contributing to IBS in the general population (**paper IV**).
- Targeted analyses, including focused genotyping of case-control cohorts for validation of GWAS findings (**paper II**), as well as searching for rare, high-penetrance variants in specific genes (**paper III**).
- Functional characterization of identified risk genes and variants (**paper III**).

3 METHODS AND MATERIALS

To facilitate better understanding for the reader of why and how our group performs genetic studies on IBS, relevant concepts, methodologies and our approach have been described already above, embedded in the section **1.5** (*Genetics of IBS*) in relation to previous work and the challenges that this complex phenotype poses.

Specific descriptions of the methods and materials used in the studies included in this thesis are fully described in the corresponding **papers I–IV** and related supplementary information.

4 RESULTS AND DISCUSSION

This section provides a summary of the main findings of this thesis, together with interpretation and discussion of results. For further information, see corresponding papers.

4.1 PAPER I

NPSR1 polymorphisms influence recurrent abdominal pain in children: a population-based study.

Recurrent abdominal pain (RAP) is the cardinal symptom of IBS and occurs frequently in children.[198-200] Compared to the adult classification system for FGIDs (Rome III), where subgrouping is based on anatomical location of the main symptoms, pediatric FGIDs are instead grouped based on predominant symptom type.[201] In children/adolescents, IBS is found in the group of 'abdominal pain-related FGIDs'.[202] Mechanisms and the genetic background of visceral sensation and pain are largely unknown, but among others, proposed candidate genes include different neurotransmitters and their receptors.[74] As described above (**1.5.5**), previous evidence suggests neuropeptide S and its receptor (encoded by the *NPSR1* gene) to be involved in nociception,[181, 182] and in addition to the brain, NPSR1 expression has been reported in enteroendocrine cells in the gut.[186] Previous work from our group has shown association of *NPSR1* polymorphisms with colonic transit and sensory ratings, and also demonstrated induced expression of other neuropeptides and gut hormones upon NPS-NPSR1 signaling *in vitro*.[77, 186] Hence, this gene was a plausible candidate to investigate further for its potential genetic association also with abdominal pain.

In **paper I**, we sought to determine whether *NPSR1* genetic variability influences RAP presence in children, and exploited data from the Swedish birth cohort BAMSE (Children Allergy Milieu Stockholm and Epidemiological Study).[203] For the purpose of the study, a total of 1,744 children with available genotype and phenotype data were included, and after quality control checks 1,556 samples could be included in the association analysis. Twenty-eight single nucleotide polymorphisms (SNPs) were used to test *NPSR1* for association with recurrent abdominal pain (RAP) using logistic regression under an additive genetic model, adjusted for sex, asthma and parents' country of origin. Phenotypic information was derived from questionnaires filled in at 12 years of age, and RAP was defined as recurrent abdominal pain occurring at least once a month. The prevalence of RAP was 9% in the study sample, with significantly more girls (70%) than boys (30%) affected (p=0.0001). Seven *NPSR1* SNPs were significantly associated with RAP, five of which withstood false discovery rate (FDR) correction for multiple testing (best p=0.00054, FDR corr. p=0.014, OR:1.55 for rs2530566). The 28 tested variants span over the entire (~220 kb) *NPSR1* gene, and the associated SNPs are all non-coding and map in a putative regulatory region upstream of the gene; hence, these may potentially exert their genetic effects through modulation of NPSR1 (mRNA) expression. In line with this hypothesis, inspection of ChIP-seq data from the Encyclopedia of DNA Elements (ENCODE) showed dense binding of transcription factors at this locus, including STAT3, CEBPB, c-Fos and others.

Although expression of NPSR1 is absent or extremely low in most human tissues, mRNA expression in several brain areas has been documented, such as the hypothalamus and amygdala.[204, 205] To further characterize potential functional consequences of *NPSR1* genetic variation of the top associated SNP (rs2530566), we inspected publicly available mRNA expression data from the Genotype-Tissue Expression project (GTEx; wwwgtexportal.org) to look for correlations between *NPSR1* genotype and mRNA expression (eQTLs) in relevant tissues. Interestingly, a significant correlation between RAP-risk allele G and NPSR1 mRNA expression was detected in the brain region amygdala (p=0.03). This is a potentially important observation since amygdala not only plays a key role in emotions and affective states such as anxiety, depression and response to fear, but also seems to be highly involved in pain modulation and perception.[206] Interestingly, studies in rats suggest NPS to have pain-inhibiting effects through mechanisms in the amygdala.[206-208]

The *NPSR1* gene was originally described as the *GPRA* (G protein-coupled receptor for asthma susceptibility) or *GPR154*, since it encodes for an, at that time, orphan receptor associated with asthma and related traits.[187-190] However, there is also evidence of a role of this receptor and its signaling in anxiety;[183, 184] a well-known comorbidity of asthma in young people,[209] but also of RAP.[210] In the current study, we were not able to use anxiety as a covariate, since data on this had only been collected at 8 years and not 12 years in BAMSE. However, we note that previous association between *NPSR1* and anxiety in the BAMSE cohort did not involve the same SNPs or haplotype as in our current study on RAP,[183] suggesting that the association with RAP is independent from that of anxiety.

The mechanisms and pathways behind pain perception are complex.[62] Visceral pain stimuli are sensed by nociceptors in the ENS, which transmit the information up to the CNS, but the sensation can also be inhibited or facilitated on different levels along the brain-gut axis through endogenous pain modulation involving various neurotransmitters, hormones and their receptors. In addition to a direct involvement in pain modulation in the brain, there are several other ways in which genotype-driven NPSR1 changes (presumably expression levels) may potentially affect visceral pain (**figure 9**), as described more in detail in the discussion of **paper I**. One potential way could be through other neuropeptides and hormones, as previous work has shown that NPS-NPSR1 downstream signaling in an *in vitro* model can induce the expression of a number of gut hormones and neuropeptides including neurotensin, cholecystokinin, vasoactive intestinal peptide and somatostatin.[77, 186] Several of these have gut motor and secretory functions but some are also involved in pain modulation. Moreover, *NPSR1* polymorphism is associated with chronic inflammatory diseases such as IBD[191] and rheumatoid arthritis,[192] and higher mRNA levels have been observed in inflammation (IBD),[191] activated macrophages and T lymphocytes,[185] and THP-1 monocytic cells upon pro-inflammatory cytokine stimulation.[186] At the same time, antinociceptive effects have been demonstrated from NPS-NPSR1 signaling in inflammatory pain models in mice.[182] Hence, a role of NPSR1 in inflammation may not only be a plausible explanation for the genetic associations with inflammatory diseases and asthma, but may also be of relevance for visceral pain and the association with RAP.

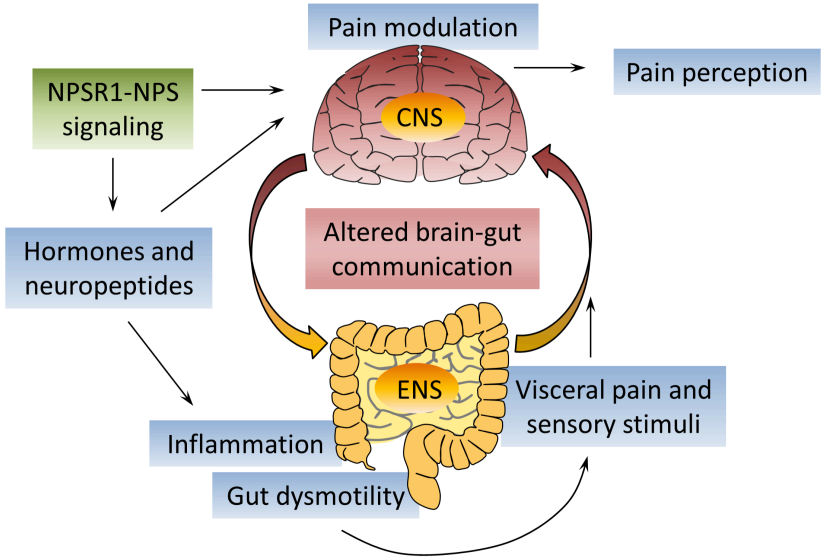


Figure 9. Schematic model for NPSR1 involvement in inflammation, gut-brain communication and visceral pain. Increased NPSR1 signaling induces the expression of various gut hormones and neuropeptides, and could directly or indirectly affect inflammatory responses and gut motor and sensory functions. CNS, central nervous system; ENS, enteric nervous system; NPS, neuropeptide S; NPSR1, neuropeptide S receptor 1. Reproduced from **paper I,** © see page 72.

The main limitation of the current study is the relatively small case sample set (159 RAP cases, 1,582 controls), which did not allow us to do *NPSR1* haplotype analyses because of insufficient statistical power. It is therefore difficult to conclude exactly which SNP or combination of variants is responsible for the observed association with RAP. In addition, our definition of RAP (1/month) does not exactly match that of Apley[211] or Rome III,[202] but the prevalence of 9% in our study is similar to that reported based on those definitions.[200, 211, 212] To confirm our results, replication and further investigations are encouraged in independent larger cohorts, and future studies should also investigate *NPS* variants and gene-gene interaction effects, since interaction of *NPS* and *NPSR1* variants have recently been demonstrated in the same cohort for asthma.[213]

In conclusion, in **paper I** we report on the involvement of *NPSR1* SNPs in the presence of RAP in 12-year-old children. Further studies are warranted to investigate the specific mechanism(s) in which NPSR1 and its signaling influence abdominal pain in FGIDs.

4.2 PAPER II

TRPM8 polymorphisms associated with increased risk of IBS-C and IBS-M.

As described in **1.5.5** above, previous work from our group in collaboration with G. Farrugia's team at the Mayo Clinic showed that 2.2% of IBS patients carry functionally relevant mutations in the ion channel gene *SCN5A*, and common SNPs also showed association in our published GWAS of IBS (also described in **1.5.5**).[56, 180] Since many ion channels are involved in mechanisms of visceral sensation and GI motility,[78] we hypothesized that also other ion channelopathies may be implicated in IBS pathophysiology.

For this purpose, association results from our GWAS[180] were inspected at genetic locations for 27 ion channel genes selected from similar functional pathways related to GI function (gut motility and visceral sensation among others, see *supplementary information* to **paper II**). This resulted in the detection of nominally significant (uncorrected $p<0.05$) association for four genes: transient receptor potential channels *TRPV3* and *TRPM8*, and the calcium voltage-gated channels *CACNA1A* and *CACNA1E*. These genes were brought out for replication and 33 tagging SNPs were selected from their loci and genotyped in an independent Swedish multicenter cohort of 386 clinical IBS cases (Rome III) and 357 controls. Sex-adjusted logistic regression analysis in this replication set detected significant associations for *TRPM8* exclusively in the constipation-predominant IBS subtypes (best $p=3.9\times10^{-5}$; OR=1.94 for rs2362290 with IBS-C and IBS-M combined), and meta-analysis of GWAS and replication data (totaling 920 cases and 5,289 controls) yielded strongest evidence of association (testing IBS overall) with no statistical heterogeneity and same direction of effects in the two studies.

The two SNPs showing strongest association and IBS risk effect in IBS-C/M map to the promoter region of the gene where the minor alleles associated with increased risk of IBS in our Swedish case-control samples. Hypothesizing that these promoter SNPs may exert their function through changes in expression levels, *in silico* analyses were performed, which predicted these two SNPs to alter transcription factor binding to DNA. Gene-set enrichment analysis (using EnrichR) of affected transcription factors yielded several significantly enriched pathways and ontologies including 'abnormal hepatobiliary system', and additional analyses also showed that *TRPM8* co-expressed genes are enriched in pathways related to bile acid/salt secretion and transport. These observations are of potential importance since bile acid metabolism/synthesis has been implicated in IBS,[51, 122] and bile acids or IBAT (the re-uptake transporter) inhibitors may be used for the treatment of constipation.[214]

Furthermore, the Population-based Colonoscopy study (PopCol) was exploited for investigating the relationship between *TRPM8* genotype and GI motility, by using stool consistency (based on Bristol Stool Form Scale [BSFS] scores from diaries) as surrogate measurement of intestinal transit time. This revealed *TRPM8* IBS-C/M risk alleles to be consistently associated with harder stools (lower BSFS scores), suggesting that these variants have constipation related effects.

The transient receptor potential cation channel melastatin 8 (TRPM8), or the 'cold and menthol receptor' (CMR1), is activated by cool temperatures from 8°C to ~28°C, covering both innocuous cool and noxious cold (<15°C) ranges (**figure 10**). Cooling compounds such as menthol, spearmint and icilin also activates the channel.[215] Cooling of the skin is a common strategy to ease pain, and it is believed that this cool-induced analgesia is mediated by TRPM8 in a 'gate control' mechanism (which implies that a nociceptive pain signal can be suppressed by an innocuous stimuli in the synaptic region of the dorsal horn).[216, 217] Interestingly, the herbal preparation peppermint oil is frequently used in IBS therapy and has shown to be superior to placebo in the improvement of global IBS symptoms and in

particular abdominal pain.[218] The major constituent of peppermint oil, menthol (a TRPM8 agonist), seems to have both antispasmodic (smooth muscle relaxing) and pain modulation properties, although underlying mechanisms are unclear.[136, 137] The TRPM8 channel appears to be implicated in visceral hypersensitivity,[82] and expression on sensory neurons innervating visceral organs, including colon, has been reported,[219] but its functions and effects do not seem that straightforward. Studies on experimental pain and knock-out models in mice have demonstrated both analgesic effects and increased pain responses from TRPM8 stimulation,[220, 221] and a role in colonic inflammation is also suggested.[82] Moreover, co-expression and cross-talk between TRPM8 and two other ion channels implicated in IBS and visceral sensation (TRPV1 and TRPA1) have been shown, and it seems like TRPM8 can inhibit their downstream chemo- and mechanosensory signaling in colonic afferent fibers through cross-desensitization.[219] Thus, taken together, TRPM8 may not only play a function in innocuous cool sensation but also in nociception as well as analgesia, depending on location and context, and it is possible that interactions and sensitizations of different TRP channels, triggered by an inflammatory state, contribute to and modulate visceral pain and hypersensitivity.[82] These TRP channels therefore represent promising drug targets for the relief of chronic pain conditions, including IBS.[82, 222]

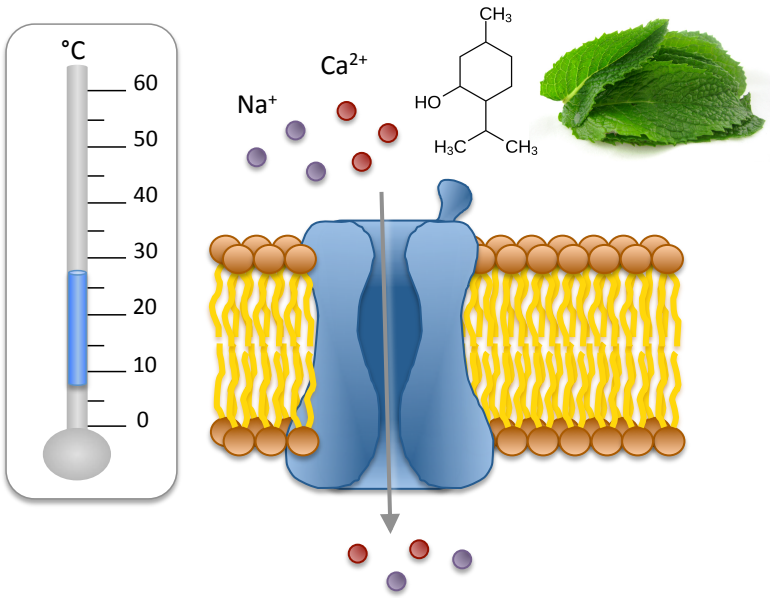


Figure 10. The transient receptor potential cation channel melastatin 8 (TRPM8) is the 'cold and menthol receptor' activated by both innocuous cool and noxious cold temperatures (ranging from 8°C to ~28°C), as well as cooling compounds such as menthol (structure shown in figure), spearmint and icilin. © See page 72 (mint leave).

The strengths with our **paper II** is the observed evidence of association in two independent study cohorts (discovery GWAS and replication case/control) that showed no statistical heterogeneity and same direction of effects, and the large sample size (total n=920 cases and n=5,289 controls). The association was stronger in the IBS-C/M group while absent in IBS-D, which suggests the genetic effect may be linked to constipation. This was also supported by the correlation between IBS risk alleles and harder stools (lower mean BSFS scores) in a

general population-based cohort. The definition of IBS used in the TwinGene population (GWAS) showed 99% concordance and high reliability (κ=0.92) with Rome II criteria.[197] However, subgrouping according to the Rome criteria was not possible with the questions available in the TwinGene study questionnaire. Thus, subgroup analysis in our study was only performed on the replication data set.

The signal of association between *TRPM8* gene and IBS detected in our study is contained in an upstream region of the gene and only includes non-coding variants. Two of the strongest IBS-C/M associated SNPs map to the gene promoter, hence, we hypothesized that their functional effect on the phenotype may be mediated through altered transcriptional control, which was also supported by the computationally predicted change from these variants in transcription factor binding affinity. However, these SNPs are in complete LD with each other and in complete or high LD with also other highly significant variants. Hence, to conclude exactly what are the causing variant(s) responsible for the association with IBS, further investigation will be needed. Of note, it is still highly possible that also rare coding variants may be of importance in certain subgroups of patients. Thus, similar to the case of *SCN5A,*[56] the *TRPM8* gene represents an ideal candidate for targeted sequencing efforts to search for rare mutations that may have a direct effect on channel function in IBS.

Taken together, in **paper II** we provide evidence of an association between *TRPM8* gene polymorphisms and increased risk of IBS with constipation (IBS-C and -M), and also observe risk alleles to correlate with lower BSFS scores (indicating slower transit time). Although further studies are warranted to evaluate causative SNPs and potential rare contributing variants, this finding adds to the increasing evidence of ion channel involvement in IBS pathophysiology, and may contribute to improved therapeutic precision in subsets of patients.

4.3 PAPER III

Functional variants in the sucrase-isomaltase gene associate with increased risk of irritable bowel syndrome.

Nutrition and dietary factors are receiving increased attention in IBS. For many IBS patients, food, and in particular carbohydrates, are well-recognized symptom triggers, post-prandial symptoms are common, especially in IBS-D, and patients tend to avoid foods perceived as problematic.[99-102, 104] Of note, carbohydrate restrictive diets have proven effective in reducing symptoms in subgroups of patients,[111, 223] and it has been suggested that at least some individuals with IBS (primarily postprandial IBS-D) may in fact suffer from carbohydrate maldigestion due to deficiency of digestive enzymes.[101]

Sucrase-isomaltase (SI) is a digestive brush-border enzyme in the small intestine, crucial for the breakdown of starch and sucrose in the final step of human digestion (**figure 11** and **4**, page 13).[106] In congenital sucrase-isomaltase deficiency (CSID), a rare genetic condition also called sucrose intolerance, mutations in the *SI* gene result in reduced or absent sucrase and/or isomaltase enzymatic activity.[224] Consequently, non-digested sucrose and partly digested starch accumulate in the intestinal lumen of these patients, who will present with osmotic

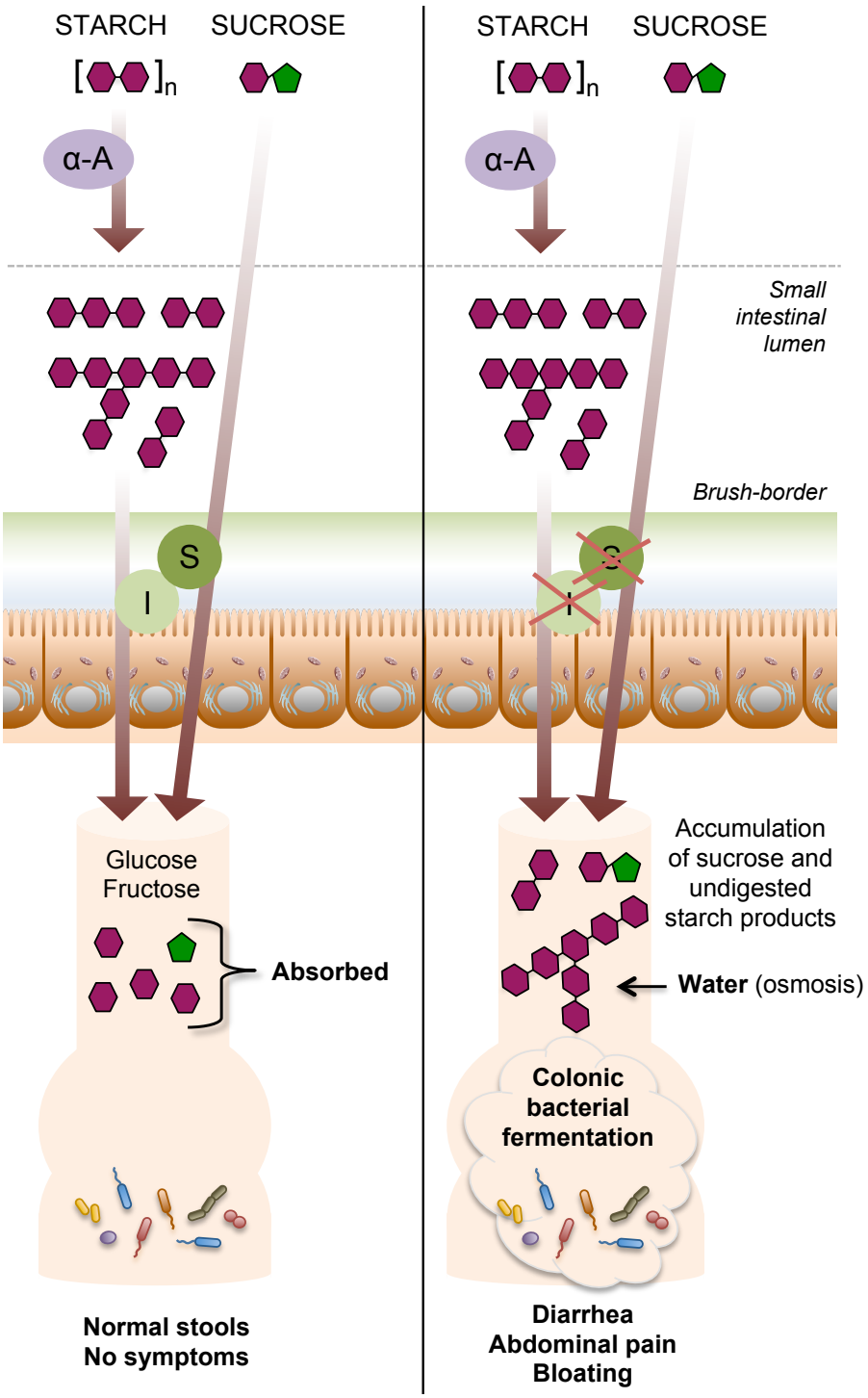


Figure 11. The role of sucrase-isomaltase (SI) and SI deficiency in starch and sucrose digestion. SI is essential for the breakdown of dietary starch and sucrose. In the brush-border of the small intestine, SI (as well as maltase-glucoamylase, not shown here) hydrolyzes α-1,4-glycosidic bonds in products of α-amylase (α-A) starch digestion. Isomaltase can also break the branching α-1,6-glycosidic bonds in isomaltose and α-limit dextrins, and sucrase hydrolyzes sucrose into glucose and fructose. In a normal state, monosaccharides are readily absorbed in the small intestine. In SI deficiency, accumulation of sucrose and partly digested (unabsorbed) starch occurs. These molecules are osmotically active, causing water retention into the lumen, and are fermented by colonic bacteria producing gas. This results in distention and increased motility, and symptoms of diarrhea, abdominal pain and bloating.

diarrhea, abdominal pain and bloating (due to colonic fermentation), —symptoms very much similar to those of IBS and chronic diarrhea. While most CSID cases are detected early in life with severe enzyme deficiencies typically caused by homozygous or compound heterozygous *SI* mutations,[225, 226] the phenotype and severity of symptoms show large variations and onset later in life has also been reported.[227, 228] This speaks for a potentially higher clinical impact of *SI* genetic variations than previously thought, and the presence of a wide range of conditions from severe to milder forms.[229, 230] Therefore, we hypothesized that *SI* mutations and/or functionally defective polymorphisms, may be relevant in IBS, in particular diarrhea.

Paper III is a multidisciplinary study where we strived to test the potential relevance of *SI* genetic variants in IBS by conducting a series of experiments and association analyses. A schematic representation of SI protein structure and the positions of the studied genetic variants are displayed in **figure 12** below, together with the key results of the study. First, sequencing of the *SI* coding region was performed in four IBS-D families including seven post-prandial IBS-D cases and one asymptomatic relative. No new *SI* mutations were identified in this effort, and all sequenced family members were homozygous for reference alleles of all common (MAF >0.01) coding (missense) SNPs, except at two sites; p.Val15Phe (rs9290264) and p.The231Ala (rs9283633). Since these, together with p.Met1523Ile (rs4855271), represent the only common coding SNPs in the *SI* gene, *in silico* analyses were performed to evaluate their potential deleterious effects. Based on computational predictions (PHRED-like scores from the Combined Annotation-Dependent Depletion [CADD] database)[231] the p.Val15Phe SNP was ranked among the top 1% most deleterious amino acid substitutions in the human genome (similar to that of CSID mutations), whereas the other two were predicted to be benign. The p.Val15Phe, which is fairly common in the general population (MAF 28% in EUR), was detected in 6/7 affected probands and segregated with IBS in the two families where *SI* sequence data of parents also were available.

Furthermore, *in vitro* experiments using COS-1 cells transfected with SI coding vectors with either Val or Phe at residue 15 provided evidence for this SNP to be functionally relevant, demonstrating the alternative 15Phe variant to be 1) 43% less associated with lipid rafts, 2) 20% less localized to the cell surface, and 3) corresponds to a 35% reduced SI enzymatic activity, compared to 15Val. These results make sense, since the p.Val15Phe is located in the stalk region of the protein, where it is highly plausible that the substitution of the aminoacid valine with a bulky phenylalanine in the first α-helix of this transmembrane domain may have an impact on the way SI associates with membranes. Consequently, fewer enzymes will end up on the cell surface of the enterocytes and SI enzyme activity overall will be impaired.

Next, together with the four most well known CSID mutations,[232] the p.Val15Phe SNP was genotyped in a multicenter cohort of 1,031 IBS cases and 856 controls from clinical centers in Europe and USA (all unrelated, and of self-reported European ancestry/'white'). Results revealed that CSID mutations were found more often in IBS patients than controls (2.13% and 1.27%, respectively, p=0.074; OR=1.84), and this finding could be consolidated by inspecting publicly available data from a large reference panel of >30,000 sequenced

European (EUR) individuals from the Exome Aggregation Consortium (ExAC [http://exac.broadinstitute.org]) (p=0.02; OR=1.57). Interestingly, in the case-control sample, the polymorphism p.Val15Phe significantly associated with diarrhea-predominant IBS (p=0.00051, OR=1.34, IBS-D and IBS-M combined) whereas no association was detected for IBS-C (p>0.05). This finding goes well in line with our hypothesis that if SI deficiency were present in IBS, it should be of relevance primarily for diarrhea-predominant phenotypes.

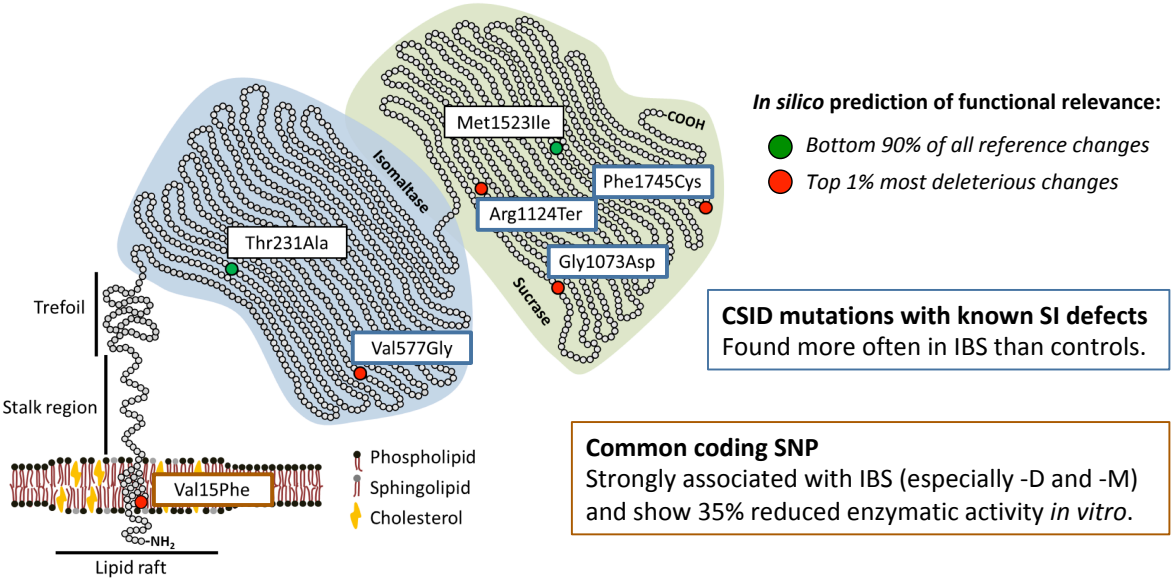


Figure 12. Schematic representation of sucrase-isomaltase (SI) protein structure and functional domains, together with the key results obtained in **paper III**. The position of the four congenital SI deficiency (CSID) mutations and the common coding *SI* polymorphisms are reported and color-coded according to their functional effects. SNP, single nucleotide polymorphism. Modified figure from **paper III**, © see page 72.

Finally, data from the general population-based study PopCol were exploited for pilot tests to investigate the p.Val15Phe variant in relation to IBS status, bowel function and microbiota composition in the general population. Although sample size was small (30 cases identified by Rome III in questionnaires, and 163 asymptomatic controls), 15Phe was significantly associated with IBS risk, in particular IBS-M (p=0.017, OR=3.81) and further strengthened the association with IBS-D/M when combined with the case-control results (p=0.00012, OR=1.36). Furthermore, diary data (totally 3 weeks daily recordings of stool patterns and gut symptoms) from 133 PopCol individuals indicated the 15Phe allele to correlate with increased stool frequency (p=0.026). Last, by testing the 20 most abundant genera in feacal microbiota data from 136 PopCol individuals, an inverse correlation was detected between the abundance of *Parabacteroides* and the number of 15Phe alleles (p=0.0024). Although this finding will have to be confirmed and we cannot draw any conclusions yet on a cause-effect relationship, it is interesting since *Parabacteroides* belong to the *Bacteroidetes* phylum and appears to be underrepresented in IBS, at least as reported in some studies.[88-90, 233]

Taken together, the results from **paper III** suggest that SI deficiency, although partial or in a milder form, is present in a subset of patients with IBS who carry heterozygous *SI* mutations.

Through a series of experiments and tests, we also provide evidence of a potential role of the common coding polymorphism p.Val15Phe, which significantly associates with increased risk of IBS, especially in the presence of diarrhea, and is functionally relevant as demonstrated *in silico* and *in vitro* with 35% reduced enzymatic activity. We thereby provide a plausible explanation of a link between carbohydrate consumption, gut microbiota and gut symptoms in a subset of IBS patients genetically predisposed to SI deficiency (**figure 13**).

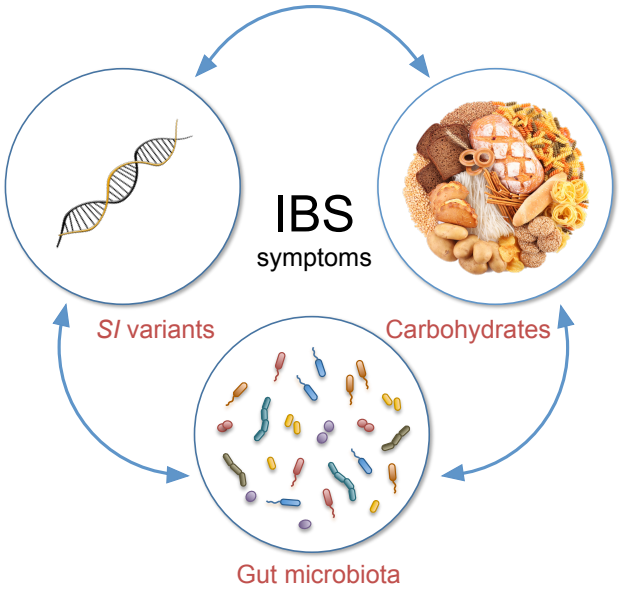


Figure 13. Interplay between genetic variation, carbohydrate consumption and gut microbiota activity. *SI* genetic variants may explain IBS symptoms in a subset of patients by predisposing to milder forms of SI deficiency. © See page 72.

The major strength with our study is the multi-disciplinary effort, where we have tried to approach our hypothesis through different levels of investigation, including sequencing in affected families, *in vitro* functional characterization, association analyses in four independent case-control cohorts, and additional pilot studies of relevant (intermediate) phenotypes in a general population-based cohort. The findings from these different levels of investigation all point to the same conclusion that *SI* genetic variation seems to play a role in IBS. In our combined case-control sample, the functionally relevant polymorphism p.Val15Phe showed strong association with IBS-D and IBS-M, and in the same study population we could also observe an almost twofold increased risk of IBS in heterozygous carriers of rare known CSID mutations (any of the four variants found in 2.13% of cases vs. 1.17% of controls). Although this finding was statistically weak, likely due to control sample size (n=1,031) inadequate to test very rare mutations, we could statistically consolidate this difference by comparing with the prevalence in the general population (>30,000 EUR individuals from ExAC), where the cumulative prevalence of these mutations was 1.37%.

Of note, this particular approach of using ExAC data as a comparison group has recently been expanded in a follow-up study from our group (published online Feb 2018, not included in this thesis).[234] In that study, a two-step computational and experimental strategy was used to determine whether also other (dys)functional *SI* variants increase the risk of IBS. First,

computational tools were used to identify clinically relevant mutations based on predicted pathogenicity scores, and a total of 880 functionally deleterious variants were identified. Thereafter, inspection of Illumina HumanCoreExome data for 2,207 IBS cases from the large ongoing *bellygenes initiative* (www.mdalab.org/bellygenes) project identified high-quality genotype data for 46 of these variants, and 17 of those were found in at least one IBS carrier and also in a reference population (n=33,370) of European ancestry from ExAC. These 17 mutations were found significantly more often in IBS cases than in the reference population (p=0.00049; OR=1.45), with a cumulative prevalence of 4.2% in IBS-D and 4.5% in IBS-C compared to 2.8% in reference controls. Hence, that study supports the results of **paper III** and provides further evidence of the prevalence of functionally deleterious *SI* variants in IBS.

To understand the specific roles of *SI* variants in IBS, functional characterization studies *in vitro* are valuable, and as recently shown by Gericke *et al.,*[235] different *SI* mutations have different biochemical and functional consequences and these authors again conclude that a spectrum appears to be present with mild to severe forms of SI deficiency. Future studies should also aim to investigate the combined effect of risk alleles, as it is possible they influence IBS through different combinations (homozygous, heterozygous or compound heterozygous). Moreover, our results provide a rationale for studies investigating the interplay between carbohydrate consumption, gut microbiota and genetic defects of involved enzymes (SI or other), as well as studies evaluating the effect from dietary modifications or enzyme supplements in IBS stratified by SI genotype. Interestingly, in another recent study from our group (published online Jan 2018, not included in this thesis),[236] utilizing two large German population-based cohorts (total n=1,398), the association of *SI* p.Val15Phe with IBS was confirmed (3.69% of 15Phe carriers reported IBS compared to 1.84% of non-carriers; p=0.044; OR=2.04) and preliminary evidence was provided on *SI* genotype-carbohydrate-microbiota interactions. The difference in IBS prevalence between 15Phe carriers and non-carriers was most pronounced in low-starch consumers (estimated from food frequency questionnaires); 7.8% vs 1.9%, p=0.029; OR=4.17. Furthermore, correlation between starch intake and microbiota composition could be observed (p=0.007), and this effect seemed to be dependent on p.Val15Phe genotype. Interestingly, taking into account also IBS status, feacal microbiota abundance of *Blautia* (known as a 'carb-digester') was significantly higher in IBS than non-IBS in the 15Phe carrier group (p=0.00041) whereas no difference could be seen in those who did not carry the 15Phe genotype (p=0.31).

To conclude, similar to lactose intolerance often presenting as IBS until diagnosis is established, milder forms of SI deficiency may be missed in this type of patient group and possibly misdiagnosed as IBS if not tested for. The results from **paper III** and others mentioned above should contribute to increased awareness of this condition as a potential underlying cause of IBS and similar FGIDs. As we learn more about the functional effects of *SI* genetic variants and their contributing role in gut symptoms, this information holds potential for eventually stratifying patients and making it possible to direct personalized treatment (i.e., dietary modifications and enzyme replacement therapy) in those with genetically derived SI deficiency.

4.4 PAPER IV

A GWAS meta-analysis from five population-based cohorts implicates ion channel genes in the pathogenesis of irritable bowel syndrome.

As described in section **1.5** (*Genetics of IBS*) above, previous studies on the genetics of IBS have been focused on single genes in hypothesis-driven approaches conducted in relatively small case-control sample sets, and most of the findings have not been adequately replicated in independent cohorts. The complex and heterogeneous phenotype of IBS poses significant challenges for those trying to decipher its genetic background, and in order to overcome some of those challenges we recently proposed an alternative strategy for IBS gene discovery. This strategy means we shift gear to a general population-based approach exploiting existing genetic and epidemiological data from large cohorts for the purpose of performing large-scale, hypothesis-free IBS genome-wide association studies (GWAS) and their meta-analyses, and has been further introduced in **1.5.5** above and in our reviews from 2013[4] and 2016.[5] This way of approaching the challenging task of exploring the genetic architecture of IBS had not been attempted before, and following our proof-of-principle pilot GWA study conducted in the Swedish TwinGene cohort,[180] we continued this work by combining it with additional independent GWA studies from Europe in a large meta-analysis of IBS.

In **paper IV**, we utilized data from five large epidemiological population-based cohorts, namely TwinGene (Sweden), LifeLines-DEEP (The Netherlands), Study of Health in Pomerania (SHIP)-Trend (Germany), TwinsUK (UK) and the Northern Finland Birth Cohort 1966 (NFBC1966, Finland), together comprising a set of 18,659 individuals with available genotype and phenotype data. Short descriptions of these cohorts are provided in the method section of **paper IV** with related references. Imputation and quality control (QC) of data was carried out at respective sites for each cohort, followed by individual GWASs using sex-adjusted logistic regression under an additive genetic model. By using questionnaire data compatible with Rome III criteria, a total of 1,335 IBS cases (74.8% females) and 9,768 asymptomatic controls (55.6% females) were identified in these five cohorts. For details on methods and computational strategy, please see **paper IV** with supplementary information. However, an overview of the study steps is provided in **figure 14**. Due to ethical restrictions in individual level genotype data sharing, QC and GWA analyses for three of the cohorts were run locally by collaborators, whereas two datasets (TwinGene and SHIP-Trend) were run by us at KI. Following the GWASs, summary statistics were quality controlled, harmonized and combined in a meta-analysis using the fixed-effect model weighted by inverse-variance. Finally, a total of 2,483,385 high-quality SNP markers could be included in the meta-analysis (passing QC and with summary statistics available from at least two datasets).

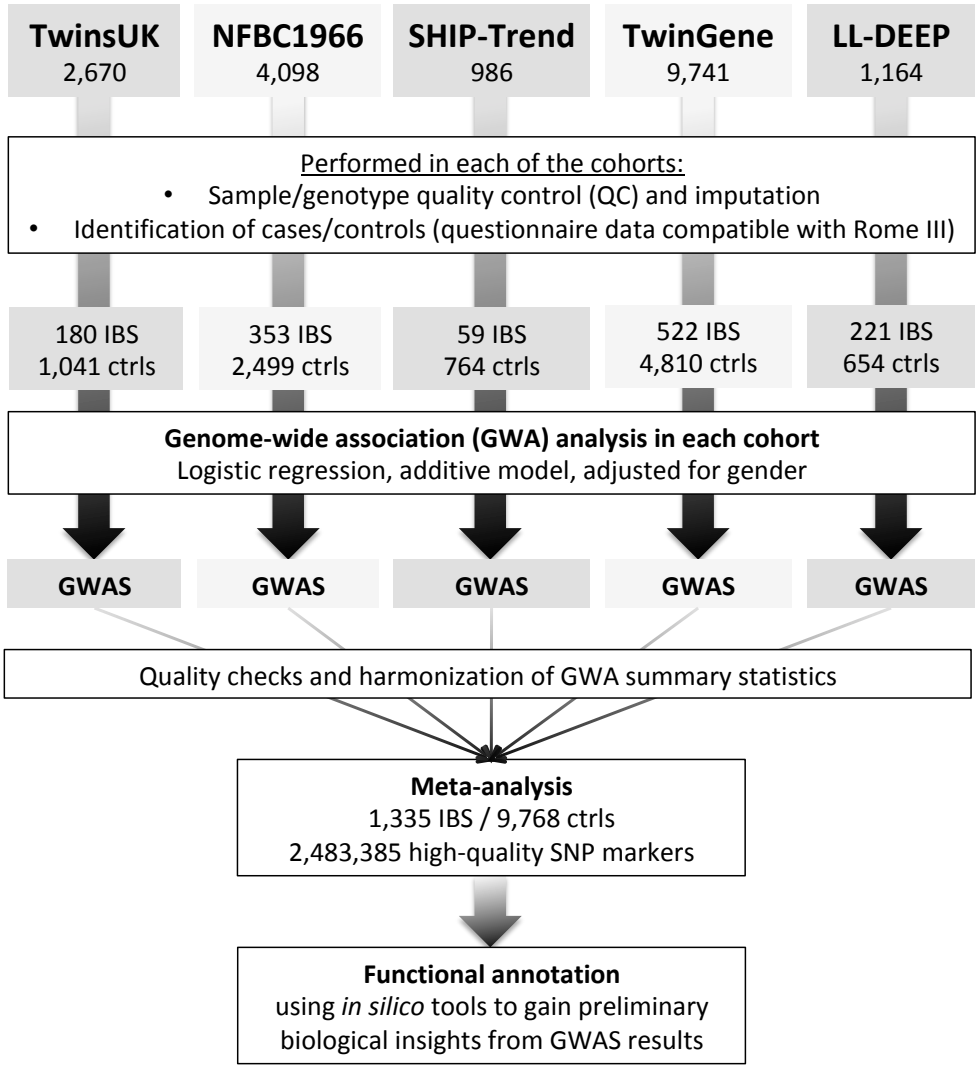


Figure 14: Flowchart of the study steps in paper IV. NFBC1966, Northern Finland Birth Cohort 1966; SHIP, Study of Health in Pomerania; LL, LifeLines.

Although no genetic marker reached genome-wide significance ($p \leq 5.0 \times 10^{-8}$) in either individual GWASs or the meta-analysis (**figure 15**), suggestive association signals ($p \leq 5.0 \times 10^{-6}$) were detected for seven genomic regions/loci in the meta-analysis with concordant genetic risk effects across studies. These loci together harbour a total of 35 genes that physically map to the regions. Worthy of mention among these genes is the *SCN1B* gene located in a suggestive risk locus on chromosome 19 and which interestingly codes for a β-subunit of the $Na_V1.5$ ion channel. As described in the introduction, the α-subunit of the same channel is encoded by the *SCN5A* gene, in which missense mutations have been identified in 2.2% of IBS patients.[56] Both *SCN1B* and *SCN5A* have been implicated in cardiac arrhythmia syndromes, such as Brugada syndrome,[237] hence *SCN1B* represents a plausible candidate IBS risk gene and deserves attention in follow-up studies. Moreover, the same loci also contains other potentially interesting genes, including FXYD(Phe-x-Tyr-Asp)-domain containing ion transport regulators (*FXYD1, FXYD3, FXYD5* and *FXYD7*), which interact with and regulate the function of ion transporters such as the Na^+/K^+-ATPase in a tissue-specific manner, affecting for example neuronal excitability or muscle contraction.[238]

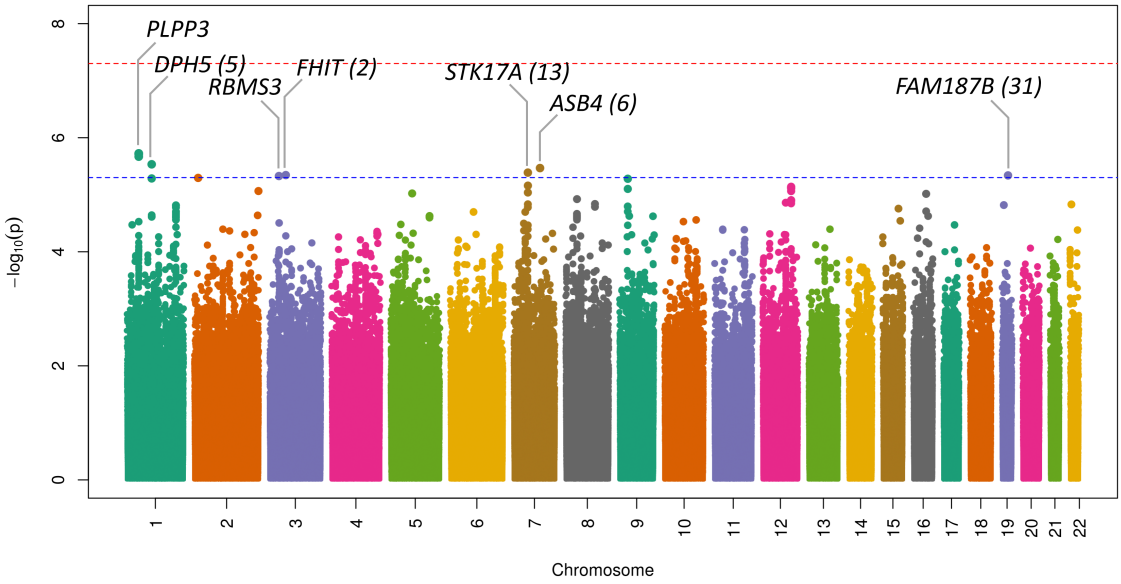
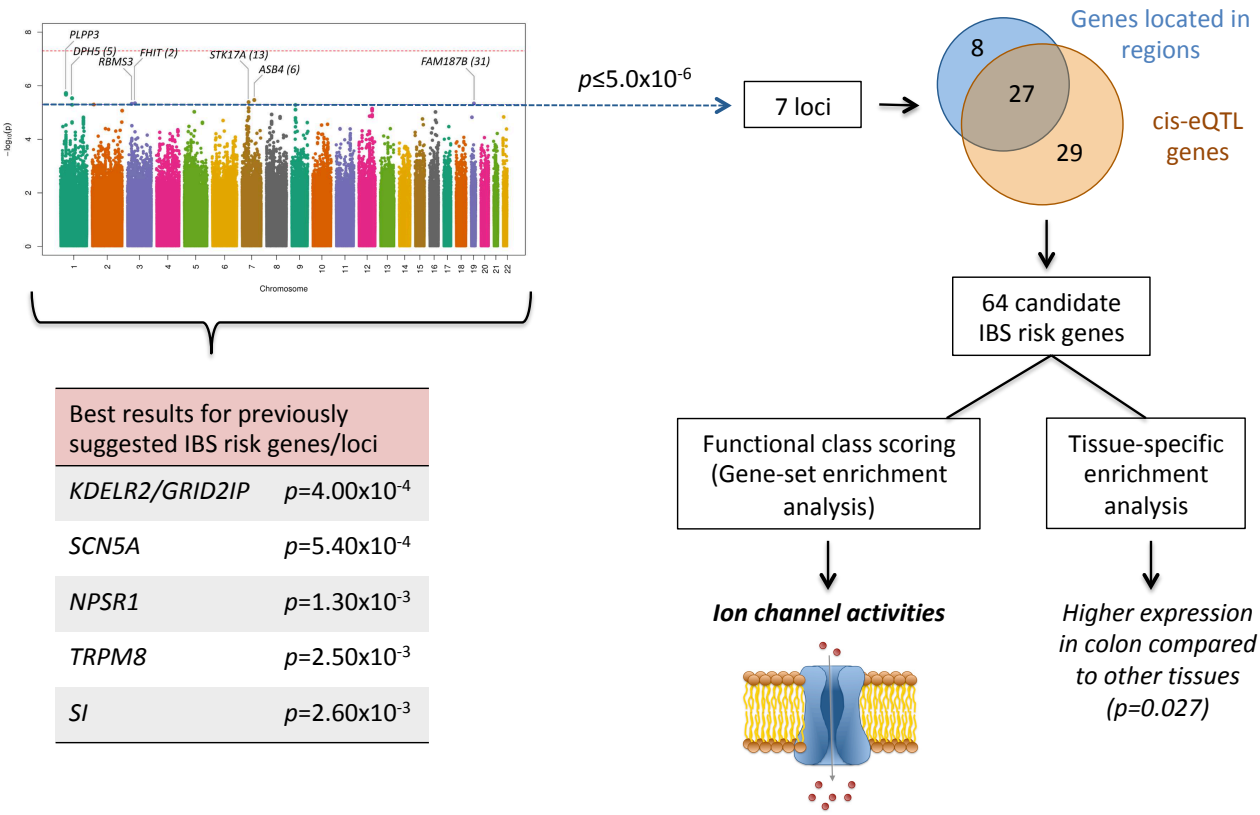


Figure 15: Manhattan plot of IBS GWAS meta-analysis results. Red horizontal line: genome wide (p=5×10^{-8}) significance level. Blue horizontal line: suggestive (p=5×10^{-6}) significance level. For each suggestive association signal, the nearest gene (mapping closest to the lead SNP) is reported, together with the number of additional genes from the same locus (in brackets). Reproduced from **paper IV**, © see page 72.

As seen in **figure 16**, the expression of most of the genes (27/35) located in the seven suggestive IBS risk loci (including *SCN1B* and the *FXYD* genes mentioned above) appears to be influenced by genetic variation in these regions (defined as cis-eQTLs), as well as an additional 29 nearby genes (identified from screening the GTEx database, version v6),[153] thereby totaling 64 potential IBS risk genes from this meta-analysis. This set of 64 candidate IBS risk genes was brought forward for further functional annotation in order to get preliminary biological insights from the observed associations using *in silico* computational tools. First, tissue-specific enrichment analysis (TSEA) results indicated higher expression (nominally significant p=0.027) of these genes in the colon compared to other tissues. Second, gene-set enrichment analysis (GSEA) returned strongly significant results for Gene Ontology (GO) terms of ion channel activities (both at the *molecular function* and *biological process* levels), which means that the 64 candidate IBS risk genes from the 7 most associated regions are enriched in ion channel related pathways. This finding is especially noteworthy since it reinforces and adds on to previous evidence from our group and others that suggests ion channel genetic variation and ion channel function contribute to IBS risk and its intermediate phenotypes (e.g., *SCN5A,*[56] *TRPM8* (**paper II**) and the GWAS of stool frequency).[84] Intriguingly, ion channel activity has recently been highlighted once again in an even larger GWAS of IBS (although using an alternative definition; self-reported doctor's diagnosis) conducted by our group, utilizing data from the UK Biobank (see further info below).[239] Moreover, in our GSEA downstream analysis of GWAS meta-analysis results, an alternative functional classification was also used; the Molecular Signatures Database (see methods in **paper IV**). Again here the *ion channels* category returned most significant, although together with enrichment of genes also in the *Liver genes - metabolism and*

xenobiotics category. This is notable since both of these biological pathways were also highlighted in the GWAS of stool frequency mentioned above.[84]



Best results for previously suggested IBS risk genes/loci	
KDELR2/GRID2IP	p=4.00x10^{-4}
SCN5A	p=5.40x10^{-4}
NPSR1	p=1.30x10^{-3}
TRPM8	p=2.50x10^{-3}
SI	p=2.60x10^{-3}

Figure 16: Summary of post-GWAS analyses based on IBS GWAS meta-analysis results. For explanation, see the text. eQTL, expression quantitative trail loci.

While conducting this, —so far the largest—, meta-analysis of IBS GWASs, we sought to also verify whether any of the IBS candidate risk genes previously reported in the literature show association in this data. Therefore, meta-analysis results were inspected in relation to 18 selected loci previously proposed to affect IBS risk or its intermediate phenotypes. Nominal significance (at least one SNP marker with p<0.05) was detected at 12 of these loci, with best evidence of replication from the *KDELR2/GRID2IP* locus (7p22.1 region detected and replicated in our pilot GWAS),[180] and the genes *SCN5A, NPSR1, TRPM8* and *SI* (**figure 16**). While association at the *KDELR2/GRID2IP, SCN5A* and *TRPM8* was expected since these were originally detected in the TwinGene sample, which largely contribute to the current meta-analysis, the signals from *NPSR1* and *SI* may represent *bona fide* replications. Considering the subtype specific effects detected for, among others, *TRPM8* in constipation-predominant IBS (**paper II**) and *SI* in diarrhea-predominant IBS (**paper III**), it would have been interesting to further test the association stratified by subtypes also in the current data. However, this was not possible as the specific questions needed for classifying subjects into Rome III defined IBS subtypes were not included in the questionnaires from all of the five cohorts. However, future studies are encouraged to investigate also subtype-specific effects.

Our previously published pilot IBS GWAS in TwinGene[180] and this meta-analysis of five GWASs represents important progress in the gene-hunting efforts conducted in IBS so far, as

these are conducted in larger sample sizes than reported before and we have been able to use an hypothesis-free approach testing common genetic variation across the entire genome. These studies corroborate our hypothesis of general population-based cohorts to provide excellent opportunities for the study of the genetics of IBS, although our current sample size (1,335 IBS and 9,768 controls) still does not seem to be large enough in order to reach genome-wide significance. However, international collaboration and the launch of *the bellygenes initiative* (www.mdalab.org/bellygenes, PI Mauro D'Amato and co-PI Alexandra Zhernakova), hold promise to overcome this challenge. This project has been granted access to genotype and phenotype data from several large European general population-based cohorts and biobanks, and finally has enabled statistically well-powered and exceptionally large-scaled genetic studies (total target population >800,000 Europeans). In fact, as mentioned above, an important next step in our general-population approach very recently got accepted for publication (April 2018) in Gastroenterology; a GWAS of IBS utilizing data from the large UK Biobank cohort.[239] In that study, self-reported doctor's diagnosis of IBS was derived from questionnaires and a (finally sufficiently powered) GWAS was performed with more than 7 million high-quality SNPs in 9,576 IBS cases and 336,499 controls. A genome-wide significant association on chromosome 9 was identified (best $p=3.57\times10^{-8}$, SNP rs10512344). The same region has previously been linked to age at menarche, and interestingly a gender-stratified analysis revealed the IBS risk effect to be present only in women ($p=4.29\times10^{-10}$) but not in men ($p=0.79$). The IBS risk allele of rs10512344 also showed association with female IBS-C in clinical case-control samples from Europe and the US ($p=0.015$) and with harder stools in women from the PopCol cohort ($p=0.0012$). Moreover, an additional 13 loci of suggestive significance ($p<5.0\times10^{-6}$) were identified in the UK Biobank GWAS, and interestingly, gene-set enrichment analysis of candidate IBS risk genes linked to these loci suggest these genes to be significantly enriched in ion channel activity related pathways. This finding is intriguing, since even though an alternative definition of IBS was used in this study —self-reported doctor's diagnosis in contrast to Rome criteria used in **paper IV—** the results from this study adds on to the emerging evidence (including that from **paper IV**) of a role of ion channels in IBS pathophysiology. In addition, it confirms the applicability of using large general-population based cohorts for the discovery of IBS risk genes and loci, and also open up for further investigation of gender specific effects in IBS.

To conclude, in **paper IV** we conducted a meta-analysis combining five independent GWA studies of data from European epidemiological cohorts. We were able to include a total of 1,335 IBS cases and 9,768 asymptomatic controls, and our results 1) demonstrate the feasibility of our alternative approach of using general population-based cohorts for the purpose of IBS gene discovery, 2) identify seven suggestive IBS risk loci that deserves attention in follow-up validation efforts, and 3) highlight ion channels and their activities as plausible biological pathways in IBS pathophysiology, which reinforces our previous observations and warrants further investigation.

5 CONCLUDING REMARKS AND FUTURE OUTLOOK

In an attempt to shed light on the so far poorly understood functional GI disorder *irritable bowel syndrome* (IBS), the overall aim of the work presented here was to identify, validate and functionally characterize the genetic component of this condition, with the scope of this thesis mainly focusing on the discovery phase. IBS is a complex and heterogeneous phenotype that poses many challenges when trying to decipher its genetic background, and importantly, we believe that this is not simply one single condition, but a constellation of disorders with similar symptoms but possibly different underlying pathophysiological mechanisms. Therefore, different study approaches have been adopted depending on the specific hypotheses investigated.

In **paper I**, using a candidate-gene approach, we report common genetic variation in the *NPSR1* gene to influence the predisposition to recurrent abdominal pain (RAP) in Swedish children, possibly through altered expression levels. This gene encodes the receptor of neuropeptide S, a signaling peptide that appears to act along the brain-gut axis to regulate various functions including pain perception, inflammation, gut motility, sensation and secretion of gut hormones and neuropeptides. The exact role of *NPSR1* and its ligand in RAP and IBS is not known and further investigation is warranted.

While IBS appears to be multifactorial and polygenic in most cases, also smaller subsets of patients seem to exist, for whom single-gene abnormalities have high penetrance on disease risk and may principally account for most of their GI symptoms. A perfect example of this is the *SCN5A* gene, an ion channel gene involved in the generation of slow waves underlying motility patterns in the gut; 2.2% of IBS patients carry functionally disruptive *SCN5A* gene mutations.[56] This observation led us to investigate also other ion channel genes of potential relevance for IBS. In **paper II**, by inspecting association data from our previously published GWAS of IBS[180] at genetic locations for 27 ion channel genes, we could provide evidence of an association with the transient receptor potential cation channel gene *TRPM8* (the 'cold and menthol receptor'). Polymorphisms in this gene showed nominal significance in the GWAS data and were significantly replicated in an independent Swedish multicenter sample where the genetic effect appeared to be specific to constipation. Confirmatory, risk alleles also correlated with harder stool consistency in a general population cohort. Thus, **paper II** provides evidence for an association of the ion channel gene *TRPM8* in IBS and constipation. The findings warrant further investigation, and the *TRPM8* gene represents an ideal candidate for future sequencing efforts in IBS to evaluate the potential relevance of also rare variants.

Dietary carbohydrates are recognized in many IBS sufferers as symptom triggers, and avoidance of specific foods perceived as problematic is common. Interestingly, >50% of 1,242 IBS patients completing a survey in the US in 2007 believed IBS is a result of *"Lack of digestion enzymes"*.[118] This was a priori designated a misconception, but in **paper III** we provide evidence of a role of sucrase-isomaltase (SI) genetic variation in IBS. The findings suggest milder forms of SI deficiency may be present in a subset of IBS patients, and in

addition to rare heterozygous mutations, we demonstrated a role of a common coding polymorphism, which significantly associates with increased risk of diarrhea-predominant IBS phenotypes, and is functionally relevant with 35% reduced enzymatic activity. Hence, genetically derived SI deficiency may explain the link between food ingestion and IBS-like symptoms in a subset of patients. If these patients can be identified, dietary modifications and/or enzyme replacement therapies are available for their symptom management.

Finally, in **paper IV** we conducted a meta-analysis combining five independent European GWA studies including a total of 1,335 IBS cases and 9,768 asymptomatic controls. This study represents an important step in our *general population-based approach* (described in **1.5.5**); a 'thinking outside the box' strategy for the discovery of IBS risk genes and variants by taking advantage of the high prevalence of IBS in the general population and the extensive amount of genotype and phenotype data available in large epidemiological cohorts. Although no genome-wide significant association was observed in our study, we identified seven suggestive IBS risk loci and highlighted ion channel activities as plausible biological pathways implicated in IBS. This study demonstrates the feasibility of the general population-based approach for the purpose of IBS gene discovery, providing a first solid foundation for similar efforts in even larger sample sizes. Through international collaboration and *the bellygenes initiative* that we coordinate (www.mdalab.org/bellygenes), a critical mass of general and dedicated resources has finally been gathered, including large European population-based cohorts/biobanks (UK Biobank, LifeLines, EGCUT, HUNT and others) and clinical case-controls from European/US tertiary centers, now enabling very large-scale genetic studies of IBS. These studies are ongoing, and excitingly, one of them just got published (available online on April 5th 2018).[239] In that study, data was utilized from the UK Biobank for 346,075 individuals (including 9,576 cases with self-reported doctors' diagnosis of IBS), representing the largest GWAS effort in IBS to date. A genome-wide significant signal was detected that had a strong effect on IBS risk in women only, and once again, post-GWAS *in silico* analyses of the genes linked to suggested IBS risk loci suggested ion channel activities to be implicated in IBS, this despite the alternative IBS definition used (self-reported diagnosis, in contrast to Rome III in **paper IV**).

In future efforts, large-scale well-powered studies hold promise to finally identify unequivocal risk loci in IBS. Findings coming from these efforts then need to be validated through replication in well-characterized IBS cases with a clinical diagnosis from specialized clinics and asymptomatic controls, and genes/variants also functionally characterized for their role in IBS. In addition, the use of alternative definitions and intermediate phenotypes of IBS will provide important information, and one should also strive to evaluate subtype and gender specific effects across different populations. To identify subgroups of patients where rare variants/mutations in single genes may be responsible for their IBS symptoms, sequencing of specific genes in well-selected cases and controls is necessary, and eventually even next-generation sequencing efforts of the entire exome or genome. Finally, although challenging, gene-gene interactions and their interplay with environmental factors will eventually need to be clarified as complex traits such as IBS result from a combination of 'nature and nurture'.

A major take-home message from this thesis is that several pieces of evidence now coming from different studies/approaches (including the studies on *SCN5A*,[56] *TRPM8*/**paper II**, GWASs of IBS defined by Rome III criteria/**paper IV**, GWAS of stool frequency,[84] and the recent GWAS of self-reported IBS in the UK Biobank)[239] clearly point to a role of ion channels and their activities in IBS. This warrants further investigation and may be of particular importance for therapeutic exploitation, as the accessibility of ion channels on the cell surface makes them attractive drug targets. Novel therapeutics may be developed, but also available agents targeting specific ion channels could be exploited for the purpose of treatment in IBS subgroups. Examples of this already exist, for instance with *mexiletine*, an anti-arrhythmic drug targeting the $Na_V1.5$ channel, which improved stool frequency in a severely constipated patient carrying a loss-of-function *SCN5A* mutation in a proof-of-principle study.[56] Following up on **paper II**, it may also be interesting to (re)evaluate the effect of peppermint oil on symptom improvement in IBS-C, and investigate potential pharmacogenetic effects, i.e., differences in response based on patients' *TRPM8* genotype.

IBS is a perfect example of a group of patients where targeted research towards individualized medicine will be of great importance. Research on IBS pathophysiology has provided an overall picture of various factors involved, with IBS summarized as a *disorder of gut-brain interaction*.[53] However, the etiology and mechanisms in this picture is still far from clear. A better insight into the genetic background of IBS will be of great help, not only to understand more of the involved mechanisms and pathways, but also to identify therapeutic targets, and importantly, contribute to the re-classification of IBS into novel subtypes (integrating different layers of data including genetic variation) based on molecular patterns and an underlying biology rather than the clinical symptomatology. Although IBS is not a 'dangerous' condition, it is still serious, and often chronic, difficult to treat and reduces quality of life for a substantial proportion of the general population. With an estimated worldwide IBS prevalence of around 10%, this corresponds to remarkably 33 million affected people in the US alone, and 74 million in Europe. Thus, as an example of the significance of genetic studies in IBS, if or when a small portion of this condition can be explained by a genetically derived dysfunction, like for instance SI deficiency or $Na_V1.5$ ion channelopathies in at least 2% of patients, then this will have implications for as many as 650,000 people in the US and almost 1.5 million people in Europe, including ~20,000 in Sweden.

To conclude, it is a long, winding and challenging road to the identification of unequivocal IBS risk genes and variants. However, I believe the studies presented in this thesis represent an important step in the right direction, moving towards an improved knowledge of IBS pathophysiology at the molecular level. As we learn more about its genetic background we will be able to distinguish underlying conditions now gathered under the same 'umbrella' of IBS and contribute to a better understanding of the etiology behind IBS symptoms. The results from these and future efforts are expected to contribute to patient stratification and improved diagnostic and therapeutic management in a more individualized manner.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Vad är IBS? Irritable bowel syndrome (IBS), colon irritabile, känslig tarm eller stressmage, —ja, kärt barn har många namn—, är idag den vanligaste typen av funktionella magtarm-besvär och drabbar mer än 10 % av befolkningen. Magbesvären är vanligare hos kvinnor än män och yttrar sig som återkommande smärta eller obehag i nedre delen av buken och avföringsrubbningar såsom diarréer (IBS-D, *diarrhea*), förstoppning (IBS-C, *constipation*) eller en kombination av både och (IBS-M, *mixed*) (**bild 1**, sid. 2). Uppblåsthet och gasighet är också vanligt. Termen *funktionell* betyder i detta fall att en medicinsk undersökning inte kan ge någon organisk eller strukturell förklaring till magbesvären (exempelvis en inflammation eller skada i tarmen) och det finns i nuläget inga biologiska markörer eller specifika tester för att fastställa IBS. Därför används istället symptom-kriterier (s.k. Rome-kriterier) för diagnosticering, i kombination med uteslutning av andra potentiella orsaker som kan yttra sig med liknande symptom, såsom glutenintolerans eller inflammatorisk magtarmsjukdom.

Vad vet vi om orsaken? Ett ökat forskningsintresse inom IBS har gjort att vi nu har en uppfattning om många olika faktorer som verkar spela roll i dess bakomliggande patofysiologi (sjukdomsmekanismer), bland annat psykosociala aspekter, tarmfloran, funktioner av tarmens egna muskler, immunsystem och nervsystem, samt dess kommunikation med hjärnan. En översiktlig bild av dessa säger oss att samspelet mellan hjärna och tarm av någon anledning är ur balans, och IBS kallas därför även för en *disorder of gut-brain interaction* (**bild 3**, sid. 10). Trots detta är etiologin (orsaken) bakom IBS fortfarande oklar och verkar kunna variera från individ till individ, så det finns tyvärr inget generellt botemedel, utan behandlingen är symptombaserad och individuell där patienten får testa sig fram. Detta gör att livskvalitén ofta är markant försämrad hos en person med IBS. Det finns även en stark koppling till stress, depression, andra typer av psykologiska och smärtrelaterade tillstånd, samt andra funktionella magtarmsjukdomar, och frånvaro från jobb/skola på grund av IBS är extremt vanligt. Därför är IBS, trots att det i sig inte är en farlig sjukdom, ändå ett mycket allvarligt problem, både för de drabbade men även för samhället och sjukvården då det orsakar höga socioekonomiska kostnader. Av denna anledning är det viktigt att vi försöker ta reda på mer om orsakerna till IBS, så att vi bättre kan förstå vilka som drabbas och varför, och hur vi kan förebygga och behandla det.

Varför studera genetiken bakom IBS? Flera studier har bekräftat att IBS har en ärftlig faktor och liksom många andra vanliga komplexa (multifaktoriella) sjukdomar så avgör en kombination av genetik och miljö vem som blir sjuk och på vilket sätt. I varje cell i kroppen finns en enorm uppsättning livsviktig och unik information (DNA) och tack vare att ca 0,1 % av denna ”instruktionsbok” är olika mellan två personer så har vi möjlighet att jämföra dessa genetiska variationer mellan sjuka och friska och på så sätt skaffa oss kunskap om vilka gener och varianter som påverkar risken för en viss sjukdom. Genom att kartlägga detta får vi bättre insikt i vilka processer och funktioner i kroppen som är involverade, och kan även identifiera tänkbara mål att rikta läkemedel mot. Målet för genetiska studier inom IBS är även att bidra

till bättre diagnostik och tydligare (ny) klassificering av sjukdomen för att kunna stratifiera patienter och möjliggöra mer individanpassade behandlingar och rekommendationer.

Hur ser den genetiska bakgrunden av IBS ut? Genetiska studier av många andra sjukdomar har lyckats väl och gett användbar information, men tyvärr ligger IBS-forskningen i detta område en bra bit efter. Flertalet riskgener har föreslagits i studier men än så länge, möjligtvis med ett (*TNFSF15*) eller ett fåtal undantag, finns inga övertygande evidens för dessa geners roll i IBS. En diffus och osäker definition av IBS, svårigheter att tydligt kunna mäta och veta vem som är drabbad, samt otillräckligt med fall-kontroll-studier från gastro-mottagningar gör att genetikforskning inom IBS är mycket utmanande. Dessutom är vi som forskar inom detta område övertygade om att IBS inte bara är en sjukdom utan snarare en mix av olika typer av tillstånd med olika underliggande orsaker trots liknande symptom. För de flesta IBS-patienter är patofysiologin multifaktoriell där flertalet faktorer i olika kombinationer spelar roll, men evidens pekar även mot att det finns vissa undergrupper av patienter där enstaka faktorer verkar kunna orsaka deras IBS-symptom. På liknande sätt ser den genetiska arkitekturen ut att vara ett polygent tillstånd för de flesta patienter, där många vanligt förekommande genetiska varianter —var och en med relativt liten påverkan på sjukdomen— tillsammans bidrar till att IBS utvecklas. Samtidigt verkar det finnas mindre subgrupper av patienter där mer sällsynta varianter (mutationer) i en eller flera specifika gener —dock med högt inflytande på risken för IBS— istället kan förklara huvuddelen av symptomen (**bild 5**, sid. 20). Därav behövs olika typer av strategier för att studera genetiken bakom IBS, alltifrån hypotesdrivna kandidatgen-studier till stora genomvida associationsstudier (GWAS).

Vad innehåller denna avhandling? Mitt doktorandprojekt har handlat om att identifiera, validera och studera funktionen av genetiska faktorer som bidrar till IBS, med den största tonvikten på identifiering. Nedan följer en kort beskrivning av de fyra ingående artiklarna i denna avhandling samt vad vi huvudsakligen kommit fram till i dessa studier.

Artikel I är en kandidatgen-studie där vi såg att genetisk variation (”snippar”; *SNPs, single nucleotide polymorphisms*) i genen *NPSR1* ökar risken för återkommande funktionell buksmärta hos 12-åriga barn (n=1 741) från en svensk födelsekohort (BAMSE). Denna gen kodar för en receptor till neuropeptid S —en signalsubstans som verkar vara involverad i flertalet funktioner i *brain-gut axis*, såsom reglering av oro och stress, inflammation, tarmmotorik och uppfattning av smärta. Ingen av de associerade SNP:arna orsakar någon förändring av själva aminosyrasekvensen i proteinet, men eftersom de är lokaliserade i början av genen så skulle de kunna påverka själva uttrycket av receptorn, vilket genuttrycksdata tillgänglig online även indikerade. På vilket sätt detta påverkar buksmärtan är dock oklar, men skulle kunna ha att göra med låggradig inflammation i tarmen, utsöndring av olika magtarmhormoner och neuropeptider, eller en direkt inverkan på hur hjärnan reglerar inkommande smärtsignaler (**bild 9**, sid. 36). Vidare studier behövs för att utreda detta.

Idén till **artikel II** är baserad på en tidigare studie från vår forskningsgrupp i samarbete med forskare på Mayo Clinic i USA, där mutationer i genen *SCN5A* hittades i 2,2 % av IBS-patienter men inte hos någon av de friska kontrollerna.[56] *SCN5A* kodar för en jonkanal som

inte bara har roll i pacemakerfunktionen i hjärtat utan även för motoriken i magtarmkanalen. Vi hypotiserade då att även andra jonkanaler relevanta för magtarmfunktioner skulle kunna ha en roll i IBS. I **artikel II** sökte vi därför först igenom resultaten från vår tidigare publicerade GWAS av IBS[180] och gick sedan vidare med fyra gener som visade association i GWAS-datan och genotypade SNP:ar från dessa gener i 386 IBS-patienter och 357 kontroller rekryterade från olika gastro-mottagningar runt om i Sverige. Flertalet SNP:ar i en av generna, *TRPM8,* visade signifikant association med IBS i denna replikationsstudie. Effekten var specifik för förstoppnings-relaterad IBS, och riskalleler korrelerade även med hårdare avföring i ett separat dataset (svenska studien PopCol). TRPM8 är en jonkanal som stimuleras av kall temperatur och bl.a. mentol, och verkar på olika sätt vara involverad i uppfattningen av både kyla och smärta. Den tycks även kunna interagera med andra typer av jonkanaler (**bild 10**, sid. 38). Intressant nog har pepparmyntsolja (rik på mentol) visats ha muskelrelaxerande och smärtlindrande effekt för IBS-patienter, även om mekanismen för detta är oklar. **Artikel II** visar att SNP:ar i *TRPM8*-genen påverkar risken för IBS och förstoppning. I framtida studier vore det spännande att utreda om även sällsynta variationer (mutationer) har betydelse för IBS hos vissa patienter (såsom i fallet med *SCN5A*-genen).

Artikel III handlar om en helt annan aspekt av IBS; spjälkning av kolhydrater. Kopplingen till mat i IBS är omöjlig att undgå; de flesta drabbade anser att olika livsmedel triggar deras symptom, vilka ofta uppstår eller förvärras efter en måltid, och det är mycket vanligt att undvika livsmedel som uppfattas som problematiska. Kolhydrater som ej absorberas i tarmen har en förmåga att dra åt sig vatten genom osmos, och fermenteras ofta av tarmbakterier i tjocktarmen varpå gaser bildas. Hos vissa kan detta orsaka diarré, utspänd tarm och obehag eller smärta. Samma sak sker exempelvis hos laktosintoleranta personer, men även i andra mer ovanliga tillstånd av enzymbrist, så som kongenital sukras-isomaltas(SI)-brist (**bild 11**, sid. 40). Detta är en medfödd sjukdom som beror på nedärvda mutationer i *SI*-genen (kodar för både sukras och isomaltas som sitter ihop i ett enzymkomplex) och orsakar malabsorption av socker och stärkelse eftersom dessa inte kan brytas ner ordentligt i tunntarmen. Den klassiska formen av SI-brist upptäcks i tidig ålder men mildare former verkar existera och diagnosen har även satts senare i livet. Detta tyder på att vissa former potentiellt är "gömda" inom IBS och i **artikel III** undersökte vi därför om *SI*-mutationer och/eller funktionella SNP:ar är av relevans för IBS. Först sekvenserades *SI*-genen i personer med IBS-D från fyra familjer. Då hittades ingen mutation men däremot en intressant SNP (p.Val15Phe) som ändrar aminosyran valin till fenylalanin i den region av molekylen som sitter förankrad i cellmembranet. 15Phe-varianten verkar kunna försämra funktionen av enzymen och detta bekräftades med experiment *in vitro* som bl.a. visade 35 % minskad enzymaktivitet jämfört med 15Val. Genom att genotypa denna SNP tillsammans med fyra *SI*-mutationer (de vanligast förekommande i kongenital SI-brist) i 1 031 IBS-patienter och 856 friska kontroller från Europa och USA, såg vi att mutationerna är vanligare hos IBS än hos kontroller och p.Val15Phe starkt associerat med risk för IBS, speciellt diarré-relaterad (**bild 12**, sid. 42). Risk-allelen 15Phe korrelerade även med ökad avföringsfrekvens och tarmflorans sammansättning i data från PopCol. Resultaten från denna studie stärker alltså hypotesen om

att SI-brist verkar finnas i ett spektrum från allvarligare till mildare former, och att vissa patienter med IBS-symptom i själva verket har en mildare variant av denna enzymbrist.

Artikel IV representerar ett viktigt steg i en speciell approach vi har för att leta efter IBS-riskgener; användning av data från stora populationsbaserade biobanker och epidemiologiska kohorter för att utföra GWAS-studier (en genetisk strategi som söker efter DNA-variationer i hela genomet) och dess meta-analyser. Därmed kan vi uppnå betydligt större studiestorlek och statistisk *power* än vad man hittills lyckats med inom IBS. Innan mitt doktorandprojekt hade inga GWAS-studier utförts av IBS, men strax efter att jag registrerades publicerade gruppen den första —en pilotstudie med data från en stor svensk tvilling-kohort (TwinGene, en del av det svenska tvillingregistret).[180] **Artikel IV** är en uppföljning av denna pilot-GWAS, där vi nu utfört fem GWAS:er av IBS i olika epidemiologiska kohorter från Europa (inklusive TwinGene) och sedan kombinerat resultaten i en stor meta-analys av totalt 1 335 IBS-fall och 9 768 asymtomatiska kontroller. Även om inga associationer kunde identifieras på s.k. genomvid signifikansnivå ($p \leq 5.0 \times 10^{-8}$) hittade vi sju DNA-regioner som eventuellt kan kopplas till IBS-risk ($p \leq 5.0 \times 10^{-6}$) (**bild 15** och **16**, sid. 47, 48). Intressant nog verkar många av generna i dessa regioner koda för jonkanaler eller vara involverade i processer som rör dess aktivitet. Jonkanaler och dess funktioner har i flera andra studier kopplats till IBS, bl.a. *SCN5A*,[56] *TRPM8*/**artikel II**, den första GWAS:en av avföringsfrekvens,[84] och dessutom i en mycket nyligen publicerad GWAS av självrapporterad IBS i en betydligt större studiepopulation (9 576 fall och >300 000 kontroller från UK Biobank) utförd av vår forskargrupp.[239] Dessa studier tillsammans pekar mot att jonkanaler är viktiga i IBS, och motiverar till fortsatta utredningar av dess specifika roller för magtarmsymptom. Denna kunskap är lovande för framtida behandling eftersom jonkanalerna ofta sitter förankrade i cellmembran väl nåbara för läkemedel, och det finns redan exempel på när redan tillgängliga läkemedel använts i IBS för att modifiera dessa jonkanalers aktivitet. **Artikel IV** demonstrerar även att vår populations-baserade strategi verkar lovande och fortsatta liknande studier men i betydligt större studiepopulationer är nu genomförbara tack vare internationella samarbeten och vårat *bellygenes initiative*-projekt (www.mdalab.org/bellygenes) där en avsevärd mängd genotyp- och fenotypdata har samlats ihop både från den generella populationen (stora epidemiologiska kohorter/ biobanker) och från gastro-mottagningar (fall och kontroller för validering och replikationsstudier).

För att sammanfatta så är det en lång och utmanande väg att vandra för att lyckas hitta IBS-riskgener och dess varianter, men jag är övertygad om att studierna i denna avhandling är ett viktigt steg i rätt riktning. Denna och fortsatt forskning ger oss bättre kunskap om vad det egentligen är vi kallar IBS, och bidrar även till identifiering av specifika underliggande orsaker hos olika mindre subgrupper av de som nu samlas under samma ’tak’. Detta kommer att leda till förbättrad diagnosticering och klassificering, samt mer individ-anpassade behandlings-strategier. Dessa framsteg kommer vara otroligt viktiga eftersom även om en liten del, säg 2 %, av IBS-patienterna kan identifieras och korrekt behandlas, så motsvarar det (förutsatt att prevalensen av IBS är 10 %) så många som 650 000 människor i USA och nästan 1,5 miljoner i Europa, varav ~20 000 i vårt lilla Sverige, som skulle kunna bli hjälpta.

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Figure references and attributions

Figure 1. Rome III criteria classification of irritable bowel syndrome (IBS) and its subtypes.

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Figure 3. An overview of IBS pathophysiology.

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Figure 4. Human digestion of dietary carbohydrates in a normal state.

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Figure 5. Hypothesis of the genetic background of IBS.

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Figure 6. Schematic illustration of the hypothetical mechanism underlying association of *TNFSF15* polymorphism with IBS.

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Figure 7. The proposed general population-based approach for the discovery of IBS risk genes and variants.

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Figure 8. Forest plot showing the association between IBS and the lead SNP (rs12702514) from a suggested IBS risk locus on chromosome 7.

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Figure 9. Schematic model for NPSR1 involvement in inflammation, gut-brain communication and visceral pain.

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Figure 10. The transient receptor potential cation channel melastatin 8 (TRPM8).

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Figure 11. The role of sucrase-isomaltase (SI) and SI deficiency in starch and sucrose digestion.

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Figure 12. Schematic representation of sucrase-isomaltase (SI) protein structure and functional domains, together with the key results obtained in **paper III**.

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Figure 13. Interplay between genetic variation, carbohydrate consumption and gut microbiota activity.

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Figure 14: Flowchart of the study steps in paper IV.

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Figure 15: Manhattan plot of IBS GWAS meta-analysis results.

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Figure 16: Summary of post-GWAS analyses based on IBS GWAS meta-analysis results.

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